



# IMMUNOLOGIC STUDIES IN PATIENTS WITH SUBACUTE BACTERIAL ENDOCARDITIS TREATED BY COMBINED PENICILLIN-HEPARIN METHOD

## I. SENSITIVITY TO PENICILLIN

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THIS series of studies was undertaken to investigate factors causing untoward reactions in patients who were undergoing therapy with a combination of penicillin and heparin.<sup>1-3</sup> The purpose of the present investigation was to determine whether the patients receiving intensive and prolonged treatment with penicillin developed a sensitivity to the drug, and whether they eventually acquired a sensitivity to the mold, *Penicillium notatum*, the source of penicillin.

The procedure of combined penicillin-heparin treatment of patients with subacute bacterial endocarditis consists of the continuous intravenous administration of penicillin in saline solution for a sufficient period of time to render the blood sterile. In addition, heparin, in a retarding menstruum,<sup>4</sup> is administered subcutaneously at intervals of several days, or the aqueous solution is given with the infusion of penicillin. Generally a single course of treatment is adequate, although some patients require repeated courses.

In a rapidly expanding literature on the results of penicillin therapy, there is little information pertaining to allergic phenomena due to the drug or to immunologic studies related thereto. Keefer and co-workers<sup>5</sup> mentioned the occurrence of fourteen instances of urticaria in over 500 treated patients. Its appearance or disappearance bore no consistent relationship to the administration or withdrawal of the drug. No immunologic investigations had been carried out. Lyons<sup>6</sup> stated that urticaria occurred twelve times in 209 treated patients and was associated with a temperature rise in some. Tests for cutaneous and ophthalmic sensitivity both during and after the active phase of urticaria were negative. The author, however, gave no details about the procedures which were employed. Precipitins were also absent. Dawson and Hobby<sup>7</sup> referred to three instances of urticaria in a group of 100 patients. Herrell, Nichols, and Heilman<sup>8</sup> noted the appearance of "cutaneous sensitivity" in two of 150 treated patients. These authors and others who reported the appearance of urticaria did not conduct any immunologic studies. Pyle and Rattner<sup>9</sup> described the occurrence of contact dermatitis in a medical officer engaged in the preparation of penicillin solutions. A positive patch test with penicillin was demonstrated.

For a number of reasons the group of patients under treatment with penicillin and heparin presented an ideal series on which to study the possible sensitizing effects of penicillin. They were receiving larger doses of the drug than were being administered in most hospitals where penicillin therapy was being studied. Furthermore, they were under treatment for fairly long periods of time. Of greatest significance was the fact that some of them received therapy in interrupted courses. Finally, these patients upon discharge from the hospital were directed to return for clinical checkup, and thus some of them could be tested weeks or months after therapy had been discontinued.

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## SKIN TEST STUDIES WITH PENICILLIN SOLUTION

*Control Study.—*

*Technique:* The sodium salt of penicillin\* was used. This preparation had a potency of approximately 1,000 Oxford units per milligram of the drug. Skin testing was performed by the intracutaneous method. When distilled water was used to dissolve the drug, it was found that the tests with this solution caused a stinging sensation at the injection sites. The use of physiologic saline as the diluent alleviated the discomfort.

The solutions were generally used from one to several hours after their preparation. At times a solution which had been stored in the refrigerator for several days or weeks was employed. Such solutions did not prove irritating nor did they seem to produce smaller reactions than freshly prepared ones.

At the outset a solution consisting of 200 units of penicillin per cubic centimeter was used, but later a concentration of from 5,000 to 10,000 units per cubic centimeter was found to be practical for routine testing. The color of the solution varied from a pale yellow to a light amber with different lots of the salt.

*Subjects:* When these studies were originally undertaken most of the patients receiving combined therapy were already in various stages of treatment. Several patients, however, were tested before therapy was instituted. Moreover, adequate control studies were made on both nonatopic and atopic subjects. Each control subject was tested with both penicillin solution and with physiologic saline. No ophthalmic tests were performed.

There were at least seventy-five control subjects. These included men and women hospital patients with various ailments, such as coronary artery disease, convalescent pneumonia, arteriosclerotic disease, and others. In addition, there were several patients who were under penicillin therapy for from one to three days. They were regarded as suitable controls because of the early stage of treatment.

The control group included thirty patients under treatment for various allergic complaints. Most of these subjects demonstrated positive reactions to the common allergens.

*Results:* Each test and saline control were recorded according to the degree of whealing and erythema production. Where indicated, tests were repeated one or several times.

In most instances it was found there was little variation between test and control in regard to the size of the wheal or the extent of erythema formation. The largest wheal was under a 1 plus and the strongest erythema, in or below a 1 plus. It was found that solutions made from some lots of penicillin were more irritating than others, especially those test solutions which were deep amber in color. Retesting of the subjects with solutions made from a more recently obtained lot of the drug frequently elicited essentially negative reactions. These tests thus compared favorably with those done with normal saline. No delayed reactions were found in either the nonatopic or atopic control subjects.

*Studies on Patients Receiving Penicillin Treatment.—*

*Subjects:* Twenty-three patients under active therapy were tested from one to six times, the average number of tests for the group being four. Each test was checked with normal saline. Tests were carried out either during the course of therapy or in the intervals between courses. The follow-up tests were done at intervals of weeks or months.

\*We are indebted to Mr. John L. Smith, of the Charles Pfizer Co., Brooklyn, N. Y., who supplied the penicillin used in this study.

Seven recovered patients in this group, who returned for a clinical checkup, were tested from twelve to thirty-two weeks after completion of the final course of treatment with penicillin. Also, two patients who were subsequently re-hospitalized, one for a reinfection, the other for congestive heart failure, were retested from seven to ten and one-half months after penicillin treatment had been concluded. In addition, four recovered patients, who had never been studied during active treatment, were tested from eight to twenty-two weeks after receiving their last course of penicillin therapy.

*Results:* The tests were recorded and analyzed as in the control studies. Since these tests were done with newer and, therefore, purer specimens of penicillin, there was even less tendency to produce irritation of the skin than was observed in the control studies. In no instance was there even a wheal or an erythema up to a 1 plus. In most instances the readings compared favorably with those of the saline controls. Where there was a slight increase in skin reactivity at the sites of the penicillin tests, it was considered that this finding fell well within the range of technical variation. No delayed reactions were encountered.

*Ophthalmic Tests:* One or two drops of the test solution (5,000 Oxford units per cubic centimeter) were instilled into the lower conjunctival sac of one eye. The conjunctiva was watched for at least twenty minutes for evidence of itching, edema, congestion, or lachrymation. There were no positive ophthalmic tests.

*Analysis of Cases:* An appraisal of such factors as duration of treatment, number of courses given, total dose administered, etc., will emphasize the significance of the findings obtained in this study. Of the twenty-seven patients, fifteen had received one course of treatment, six were given two courses, two had three courses, one had four courses, one was given five courses, and two received six courses. The longest period of treatment in a patient who had been given a single course was twenty-three days. The largest dose administered to a patient receiving a single course of therapy was 4,785,000 Oxford units during a twenty-one-day period. In patients to whom multiple courses were given, the longest course covered a period of fifty-two days, during which 44,500,000 units had been administered. The rest intervals between courses ranged from two to fifty-three days.

The most striking data concern a man who had received six courses of therapy over a total period of 230 days, of which 185 were actual treatment days. The courses ran from twelve to fifty-two days, the free intervals from two to thirteen days. The smallest dose in any one course was 2,700,000 units, and the largest was 44,500,000 units. The total dose given during the entire period of treatment was 112,620,000 Oxford units. This patient did not develop any clinical sensitivity to penicillin nor did he show any positive skin reactions to penicillin.

#### STUDIES FOR SENSITIVITY TO *P. NOTATUM*

*Test Materials and Procedure.*—The solution used for intracutaneous testing was an extract of *P. notatum* equal to .01 or .1 mg. total nitrogen.\* All penicillin-treated patients were tested with these solutions and were given a control test with buffered saline. Tests were checked frequently when indicated. As a further control, many of the patients, particularly those who showed question-

\*Allergenic extract for skin testing supplied through the courtesy of Dr. A. F. Coca, of Lederle Laboratories, Inc., Pearl River, N. Y.



able or small reactions, were in addition tested with extracts of other molds such as *Alternaria*, *Hormodendrum*, *Penicillium rubrum*, and *Penicillium digitatum*.

*Results.*—In many instances both test and control gave similar findings and many of these were completely negative. In others more whealing and erythema were present at the test site than at the control. Whealing was never larger than a 1 plus. The degree of erythema did not exceed a 1 plus. Furthermore, in all instances where a small reaction was elicited with the extract of *P. notatum*, reactions of similar intensity were brought out by one or usually all of the other molds.

As additional control, in order to determine the average skin reaction produced by testing with *P. notatum*, at least 200 subjects, most of them allergic patients, were tested with this extract and, in most instances, with the other mold extracts mentioned previously. Here again it was found that in the concentration .1 mg. nitrogen per cubic centimeter, the extract of *P. notatum* was essentially nonirritating to the skin and only occasionally produced a small wheal and erythema. In such instances tests with the other mold extracts elicited similar findings.

#### DISCUSSION

Twenty-seven patients undergoing prolonged intensive therapy with penicillin did not reveal any sensitivity by either intracutaneous or ophthalmic tests. The dosage of penicillin had been high, treatment had been given over long periods of time, and in nearly one-half the patients therapy had been administered in interrupted courses. These circumstances would ordinarily favor active sensitization to the drug employed, if a potent antigenic substance were involved. The results clearly indicate that penicillin is not a potent antigenic product.

Only one report in the literature calls attention to the presence of antibodies to penicillin. Criepp<sup>10</sup> demonstrated positive direct and passive transfer tests to penicillin in a patient who had developed urticaria following the first injection in a second course of treatment. Another report of interest immunologically was the demonstration by Welch and Rostenberg<sup>11</sup> of an intense, delayed, tuberculin type reaction resulting from the intracutaneous test with either commercial or crystalline penicillin in a person who had never been treated with the drug. He had been engaged for fifteen years in the handling of a variety of molds. In a footnote these authors state that approximately 5 per cent of 140 unexposed persons exhibited a tuberculin type reaction to the initial injection of crystalline penicillin sodium. This is at variance with the findings obtained in the present study, wherein no delayed reactions were obtained in either atopic or nonatopic control subjects.

The second part of this study revealed the absence of any positive reactions to an extract of the mold *Penicillium notatum*. The counterpart of these results is found in the findings of Feinberg,<sup>12</sup> who studied ten patients who were sensitive both clinically and by scratch test to the molds *P. digitatum* or *P. rubrum*. They gave positive intracutaneous tests with an extract of *P. notatum* but did not react to a solution of penicillin containing 25,000 Oxford units per cubic centimeter.

#### SUMMARY

Twenty-seven patients receiving intensive and prolonged treatment with penicillin failed to develop any positive skin or ophthalmic reactions to a solu-

tion of penicillin containing 10,000 units per cubic centimeter. These patients did not develop any positive skin reactions to the source of this product, the mold *P. notatum*.

From the present study penicillin would appear to be a very poor sensitizing agent.

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### THE EFFECT OF ARTIFICIALLY INDUCED FEVER ON HUMORAL ANTIBODIES AND ON HISTAMINE INTOXICATION IN THE GUINEA PIG

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IN A PREVIOUS report<sup>1</sup> it was demonstrated that sensitivity to horse serum in actively sensitized guinea pigs was suppressed by artificially induced hyperpyrexia. Protection was afforded when sensitized animals were heated to 43.3° C. (110° F.) and immediately injected with horse serum or when they were subjected to a temperature of 42.2° C. (108° F.) for thirty minutes and then given an intravenous injection of antigen. In this connection we have attempted to study the effect of fever on several immunologic reactions to determine, if possible, the mechanism involved in the suppression of anaphylactic shock at febrile temperatures. Since greater protection was obtained in old than in young guinea pigs, we have used, whenever possible, animals 2 to 3½ years of age.

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## EXPERIMENTAL

*The Effect of Fever on the Antiprotein Titer of the Blood Serum.*—Ecker and O'Neal<sup>2</sup> and Ellingson and Clark<sup>3</sup> have shown that fever temperatures reduce the antibody titers of rabbits immunized against *Eberthella typhosa*. The effect of fever on humoral antibodies, therefore, was investigated first since quantitative changes in antibody titer might account for a decrease in the sensitivity of an animal toward a specific antigen. For this study seven old male guinea pigs, sensitized by subcutaneous injection of 1.0 c.c. of a 1:100 dilution of horse serum three months previously, were injected intravenously with 0.1 c.c. of a 1:2 dilution of normal horse serum, followed three days later by another injection of 0.1 c.c. of the undiluted antigen. One animal died of anaphylactic shock after the first intravenous injection. The six surviving animals were subjected to high temperature from sixteen to thirty days after the final injection of antigen. One to two hours before, they were bled by incision of a marginal ear vein; they were then placed in the air-conditioned cabinet and a fever temperature of 42.2° C. was induced as previously described.<sup>1</sup> After this rectal temperature had been maintained for sixty minutes, the animals were removed from the cabinet and 0.5 to 1.5 c.c. of blood obtained by incising a vein in the opposite ear. Precipitin tests (antigen-dilution method) were made on the serums using "micro" tubes and graduated capillary pipettes. The results of the titrations are given in Table I. They indicate that fever has no influence on the precipitin titer of the serum, as measured by the antigen dilution method.

TABLE I. THE EFFECT OF A FEVER TEMPERATURE ON THE PRECIPITIN TITER OF IMMUNIZED GUINEA PIGS

PIG	PRECIPITIN TITER BEFORE FEVER TREATMENT	PRECIPITIN TITER AFTER A FEVER TEMPERATURE AT 42.2° C. FOR 60 MINUTES	TIME BETWEEN LAST IMMUNIZING INJECTION AND FEVER SESSION (DAYS)
P 2	1:64	1:64	16
P 3	1:512	1:512	17
P 4	1:64	1:64	20
P 5	0	0	21
P 6	1:64	1:64	27
P 7	1:32	1:32	30

In the following experiment an effort was made to determine the effect of fever on animals whose serum contained only small quantities of antiprotein antibody. This experiment was conducted over a period of four months, and four groups of guinea pigs sensitized at various intervals were used. They were sensitized by subcutaneous injection of 1.0 c.c. of a 1:10 dilution of normal horse serum and were employed from twenty-nine to seventy-six days after sensitization. Before using a group, four to six animals were sacrificed to obtain the approximate minimum lethal dose. As shown in Table II, the lethal dose has varied. The reason for this variation is not clear and indeed in the last group the lethal dose for the individual animals was so variable that an approximate titer could not be established. One-half of the remaining sensitized animals in each group were heated and the others were used for controls. On the day of the heat treatment the animal to be treated was bled. An hour or two later a temperature of 42.2° C. was induced and maintained for sixty minutes. Another bleeding was made and the approximate dose of antigen, as determined previously, was injected intravenously. The control animal was injected on the same day with the same quantity of normal horse serum which was injected into the heat-treated animal.

TABLE II. THE EFFECT OF FEVER ON THE SERUM ANTIPROTEIN TITER OF OLD GUINEA PIGS SENSITIZED TO HORSE SERUM

HEAT TREATED ANIMALS													CONTROL ANIMALS		
PIG	TITRATION OF SERUMS							RESULTS	DOSE OF HORSE SERUM INJECTED (C.G.)	PIG	DOSE OF HORSE SERUM INJECTED (C.G.)	RESULTS			
	SERUM DILUTION	ACCEPTOR ANIMALS		MEAN DIAMETERS OF REACTIONS*			RATIO								
		NO. INJECTED	NO. POSITIVE	SERUM OB-TAINED BEFORE FEVER	SERUM OB-TAINED DURING FEVER										
					SERUM OB-TAINED BEFORE FEVER	SERUM OB-TAINED DURING FEVER									
Group 1 JJ 511	1:4	4	4	13.3 by 16.3	12.0 by 15.0	0.83	0.75	Slight shock; recovered	0.75	S 261	0.75	Died; 3 min.			
T 574	1:4	3	3	15.0 by 18.6	14.0 by 16.3	0.82	-†	-	-	-	-	-			
Group 2 C 963	1:4	6	6	19.6 by 21.6	16.5 by 20.0	0.78	0.75	Slight shock; recovered	0.75	S 197	0.75	Died; 6 min.			
A 661	1:4	12	3	14.0 by 17.3	13.6 by 17.3	0.97	0.75	Slight shock; recovered	0.75	S 847	0.75	Died; 4 min.			
JJ 249	1:4	6	0	0	0		0.75	No symptoms	0.75	C 477	0.75	Moderate shock; recovered			
Group 3 B 205	1:4	6	5	15.0 by 18.7	14.8 by 18.7	0.99	0.875	Slight shock; recovered	0.875	O 931	0.875	Moderate shock; recovered			
B 214	1:4	6	4	21.7 by 23.5	20.0 by 22.0	0.87	0.875	Slight shock; recovered	0.875	C 371	0.875	Died; 5 min.			
A 627	1:4	6	5	23.0 by 25.0	23.0 by 26.0	1.04	0.875	Slight shock; recovered	0.875	C 522	0.875	Died; 5 min.			
A 628	1:4	6	5	26.4 by 28.4	26.8 by 28.2	1.01	1.0	Moderate shock; recovered	1.0	B 223	1.0	Died; 5 min.			
Group 4	1:8	6	5	18.4 by 20.0	18.4 by 19.2	0.96									
O 34	1:4	6	1	11.0 by 13.0	13.0 by 14.0	1.27	-			-					
D 305	1:4	6	3	16.6 by 20.3	17.6 by 19.6	1.02	-			-					
DD 658	1:4	6	6	16.0 by 18.7	17.2 by 19.5	1.12	-			-					
J 693	1:4	6	6	12.8 by 17.0	13.8 by 15.0	0.96	-			-					

\*Diameter of reactions in millimeters.

†Not done.



TABLE III. A COMPARISON OF THE SKIN REACTIONS ELICITED BY INTRAPERITONEAL INJECTION OF HORSE SERUM IN GUINEA PIGS PREVIOUSLY SENSITIZED LOCALLY WITH A 1:10 AND A 1:16.6 DILUTION OF ANTIHORSE SERUM ANTIBODY

PIG	WEIGHT (GM.)	DILUTION	READING*	DILUTION	READING*
F 193	305	1:16.6	0	1:10	0
F 193	290	1:16.6	0	1:10	0
F 144	315	1:16.6	9 by 13 by 0.5	1:10	11 by 16 by 1.0
F 157	300	1:16.6	10 by 12 by 0.5	1:10	13 by 16 by 1.0
F 182	315	1:16.6	9 by 11†	1:10	11 by 14†
J 148	290	1:16.6	11 by 14 by 0.5	1:10	12 by 15 by 0.5
J 125	300	1:16.6	10 by 13 by 0.5	1:10	14 by 18 by 0.5
J 135	305	1:16.6	17 by 19 by 1.0	1:10	21 by 23 by 2.0
J 3	295	1:16.6	13 by 15 by 0.5	1:10	17 by 20 by 1.0
J 106	315	1:16.6	0	1:10	0
J 1	315	1:16.6	0	1:10	0
AA 809	300	1:16.6	6 by 6†	1:10	9 by 10†
AA 919	295	1:16.6	12 by 14†	1:10	14 by 15†
CC 956	280	1:16.6	0	1:10	0
AA 936	300	1:16.6	9 by 14†	1:10	12 by 14†
AA 406	300	1:16.6	7 by 8†	1:10	10 by 14†
CC 934	290	1:16.6	7 by 8†	1:10	11 by 12†

Mean diameters of reactions 10.0 by 12.2 12.9 by 15.6

Ratio of areas: 0.61.

\*Diameter and elevation of reactions in millimeters.

†Erythematous reaction; no edema.

TABLE IV. A COMPARISON OF THE SKIN REACTIONS ELICITED BY INTRAPERITONEAL INJECTION OF HORSE SERUM IN GUINEA PIGS PREVIOUSLY SENSITIZED LOCALLY WITH TWO INJECTIONS OF A 1:10 DILUTION OF ANTIHORSE SERUM ANTIBODY

PIG	WEIGHT (GM.)	DILUTION	READING*	DILUTION	READING*
J 156	290	1:10	10 by 15†	1:10	9 by 14†
E 844	290	1:10	16 by 20†	1:10	16 by 20†
J 154	295	1:10	11 by 17†	1:10	12 by 17†
J 183	290	1:10	17 by 22 by 1.0	1:10	16 by 22 by 1.0
F 17	300	1:10	0	1:10	0
J 519	290	1:10	0	1:10	0
F 44	290	1:10	14 by 18 by 1.0	1:10	14 by 19 by 1.0
J 44	300	1:10	17 by 21 by 2.0	1:10	16 by 21 by 2.0
J 2	310	1:10	13 by 16 by 1.0	1:10	13 by 15 by 1.0
J 79	285	1:10	13 by 19 by 0.5	1:10	14 by 19 by 0.5
H 589	285	1:10	0	1:10	0
CC 629	310	1:10	11 by 13†	1:10	11 by 15†
AA 731	310	1:10	10 by 14†	1:10	11 by 14†
AA 789	310	1:10	0	1:10	0
AA 795	310	1:10	13 by 16†	1:10	12 by 16†
CC 708	285	1:10	0	1:10	0

Mean diameters of reactions 13.2 by 17.4 13.1 by 17.5

Ratio of areas: 1.0.

\*Diameter and elevation of reactions in millimeters.

†Erythematous reaction; no edema.

intraperitoneally are given in Table IV. The ratio obtained was 1.00, indicating that the method can be used for quantitative determination of the antiprotein titer of serums.

One-quarter cubic centimeter syringes and short beveled 27-gauge needles were used for making the intradermal injections. It was found that leakage of the diluted antiprotein serum after intradermal injection could be prevented by keeping the needle in place in the skin for from six to ten seconds after injection. Young guinea pigs were used for titration because old ones injected with the same or with more concentrated solutions of antiprotein serum intradermally did not react, even though the amount of antigen injected intraperitoneally, calculated from the weight of the animals, was proportional to that given to the young guinea pigs.

*The Effect of Fever on Histamine Intoxication.*—Since a febrile temperature did not appear to alter the humoral antibody content of the serum significantly but did reduce sensitivity toward the specific antigen, the effect of fever on a nonspecific stimulant, histamine, was studied. Histamine was chosen because practically all of the symptoms of anaphylactic shock are thought to be due to liberation of this substance during the antigen-antibody reaction.<sup>5</sup> Twenty old guinea pigs were kept in the heat cabinet at 42.2° C. for thirty minutes, and at the end of this period a dose of histamine dihydrochloride\* was injected intravenously. The dose of histamine which killed 80 to 100 per cent of the control animals was determined by preliminary titration on guinea pigs of approximately the same age and weight. As a control, one nonheat-treated guinea pig for each treated animal was injected at the same time and with the same quantity of histamine. The histamine solutions were freshly prepared each day and the concentrations reported below refer to the free base. The results given in Table V show that thirteen of the twenty heat-treated animals recovered, whereas only one of the control animals survived. For the male animals, the dose of histamine was again determined by preliminary titration on a small group. From these results it appeared that they were more susceptible to histamine and that 0.18 mg. was sufficient to kill them. Unfortunately, at that time a large number of male animals was not available to determine whether this was the case. When old boars were again available, a comparison of the toxicity of histamine in old male and female guinea pigs was made. The results, which are not given in detail, indicated that there was no essential difference in the toxicity of histamine in the two sexes.

TABLE V. THE EFFECT OF A FEVER TEMPERATURE OF 42.2° C. MAINTAINED FOR THIRTY MINUTES ON THE RESPONSE TO HISTAMINE IN OLD GUINEA PIGS

PIG	FEVERED ANIMALS			PIG	CONTROL ANIMALS		
	DOSE OF HISTAMINE* (MG.)	RESULTS	SEX		DOSE OF HISTAMINE* (MG.)	RESULTS	SEX
H 5	0.24	Died; 4 min.	F	Q 968	0.24	Died; 2 min.	F
H 6	0.24	Recovered	F	I 137	0.24	Died; 2 min.	F
A 617	0.24	Died; 6 min.	F	C 908	0.24	Died; 5 min.	F
A 697	0.24	Recovered	F	H 7	0.24	Died; 5 min.	F
H 8	0.24	Recovered	F	C 968	0.24	Died; 2 min.	F
A 674	0.24	Recovered	F	C 511	0.24	Died; 2 min.	F
H 10	0.24	Recovered	F	H 9	0.24	Died; 2 min.	F
C 980	0.24	Died; 2 min.	F	R 727	0.24	Died; 3 min.	F
H 65	0.24	Recovered	F	R 465	0.24	Died; 2 min.	F
BB 519	0.24	Recovered	F	A 647	0.24	Recovered	F
A 662	0.24	Died; 3 min.	F	U 620	0.24	Died; 2 min.	F
B 982	0.24	Recovered	F	JJ 824	0.24	Died; 2 min.	F
D 400	0.24	Recovered	F	W 922	0.24	Died; 2 min.	F
H 10	0.24	Died; 5 min.	F	H 11	0.24	Died; 60 min.	F
H 12	0.24	Recovered	F	A 697	0.24	Died; 3 min.	F
Q 765	0.24	Recovered	M	N 11	0.24	Died; 2 min.	F
H 13	0.21	Recovered	M	B 541	0.18	Died; 2 min.	M
H 14	0.18	Died; 60 min.	M	J 640	0.18	Died; 2 min.	M
H 15	0.18	Recovered	M	W 679	0.18	Died; 3 min.	M
H 17	0.18	Died; 2 min.	M	H 16	0.18	Died; 3 min.	M
Recovered		13				1	
Died		7				19	

\*Calculated as the free base.

To ascertain whether guinea pigs were protected after heat treatment and after their temperatures were allowed to return to normal, the following experiment was made. Ten old female guinea pigs were kept at a temperature of 42.2° C. for thirty minutes. At the end of this period the cabinet doors were opened and defervescence allowed to take place. After the animals attained their

\*Eastman Kodak Co., Rochester, N. Y.

prefever temperature, a dose of histamine was injected intravenously. For a control, one nontreated animal was injected at the same time and with the same dose of histamine. The results shown in Table VI indicate that the animals are protected against histamine only during the height of fever. After the temperature has returned to normal, they are as susceptible to the stimulant as are the control animals.

TABLE VI. THE EFFECT OF A FEVER TEMPERATURE OF 42.2° C. MAINTAINED FOR THIRTY MINUTES ON THE RESPONSE TO HISTAMINE WHEN INJECTED AFTER THE TEMPERATURE HAD RETURNED TO NORMAL

FEVERED ANIMALS			CONTROL ANIMALS		
PIG	DOSE OF HISTAMINE* (MG.)	RESULTS	PIG	DOSE OF HISTAMINE* (MG.)	RESULTS
C 914	0.24	Died; 1 min.	A 607	0.24	Died; 3 min.
CC 704	0.24	Died; 2 min.	BB 339	0.24	Died; 2 min.
D 235	0.24	Died; 3 min.	N 1	0.24	Died; 4 min.
JJ 880	0.24	Died; 3 min.	W 767	0.24	Died; 3 min.
JJ 871	0.24	Recovered	D 268	0.24	Died; 30 min.
HH 317	0.24	Died; 3 min.	N 2	0.24	Died; 4 min.
N 550	0.24	Died; 2 min.	N 3	0.24	Died; 45 min.
H 624	0.24	Died; 16 min.	R 739	0.24	Died; 3 min.
I 534	0.24	Died; 3 min.	B 315	0.24	Died; 3 min.
Q 317	0.24	Died; 4 min.	Q 930	0.24	Recovered
Recovered		1			1
Died		9			9

\*Calculated as the free base.

*The Response of Intestinal Strips Toward Histamine When Exposed at Various Temperatures.*—The effect of histamine on surviving intestinal strips was studied to determine whether the same temperature shown to suppress anaphylactic shock in vivo influenced the degree of contraction of this tissue. In these experiments the tissue was heated to a temperature of 43.3° C. (110° F.) since it was demonstrated that bringing sensitized guinea pigs to this temperature afforded protection against anaphylactic shock. Old normal female guinea pigs were used. They were sacrificed and as soon as reflex movement ceased, the abdomen was opened and the ileum removed and placed in warm Ringer-Locke's solution. The ileum was carefully dissected from the mesentery and cut into segments approximately 12 cm. in length. Detritus within the lumen was washed out by allowing large quantities of warm Ringer-Locke's solution to flow through by gravity. The segments were placed in separate beakers containing nutrient solution and were used immediately or stored at 5° C. until needed. Nicoll and Campbell<sup>6</sup> found that at a temperature of 5° C. ileal segments retain their activity for from three to five days. We have verified this observation. Before use, the 12 cm. segments of ileum were cut into four strips approximately 3 cm. in length and one or two of these strips suspended in a tissue bath containing Ringer-Locke's solution. Oxygen was bubbled through the tissue bath throughout the experiment. The tissue bath was suspended in a constant temperature bath regulated at 38.8 or 39.0° C. (101.8 or 102.2° F.). This temperature was 0.3 and 0.05° C., respectively, lower than the mean rectal temperature of the heat-treated guinea pigs injected with histamine. The tissues were connected to muscle levers with silk thread and recordings made as described by Dale.<sup>7</sup> Instead of smoked paper, the ink writing pointers described by Friedman<sup>8</sup> were employed. The tissue was allowed to remain in the oxygenated nutrient solution for from forty to fifty minutes before being tested with histamine. The quantity of histamine added to the muscle bath was an amount sufficient to provoke a submaximal contraction of the tissue. The tis-



sue was washed with three changes of nutrient solution having the same temperature as the bath. The temperature was then increased to  $43.3^{\circ}\text{C}$ . This usually required from forty to fifty minutes. When this temperature was attained, the same quantity of histamine was added. After washing the tissue three times, the bath was cooled to  $38.8^{\circ}\text{C}$ . by running cold water through a copper coil suspended in the bath. The same amount of histamine was then added as before. Three typical tracings are shown in Fig. 1. They indicate that there is less contraction of the tissue at  $43.3^{\circ}\text{C}$ . than at  $38.8$  or  $39.0^{\circ}\text{C}$ . Thirty-five of these experiments were made with tissue derived from six old guinea pigs. All except two of the tissues showed less response at  $43.3^{\circ}\text{C}$ . than at  $38.8$  or  $39.0^{\circ}\text{C}$ . when exposed to the same concentration of histamine. The average magnitude of the contractions after the first addition of histamine at  $38.8$  or  $39.0^{\circ}\text{C}$ . was  $13.1\text{ mm.}$ , at  $43.3^{\circ}\text{C}$ . it was  $7.0\text{ mm.}$ , and after the last addition at  $38.8$  to  $39.0^{\circ}\text{C}$ . it was  $14.4\text{ mm.}$

In other experiments the temperature was raised to  $45^{\circ}\text{C}$ . ( $113^{\circ}\text{F.}$ ) or  $46.0^{\circ}\text{C}$ . ( $114.8^{\circ}\text{F.}$ ) and the tissue exposed to histamine. Two typical tracings are shown in Fig. 2. They indicate that there is a marked decrease in response

## EXP. 38

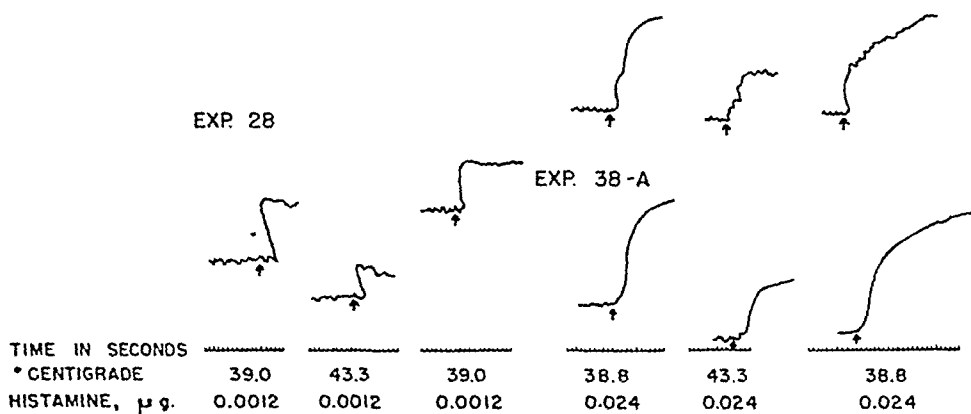
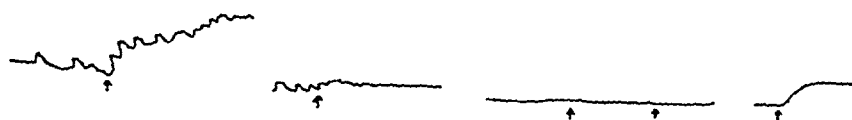


Fig. 1.—The effect of temperatures of  $38.8$  to  $39.0^{\circ}\text{C}$ . and  $43.3^{\circ}\text{C}$ . on the response of ileal strips to histamine.

## EXP. 37



## EXP. 37-A

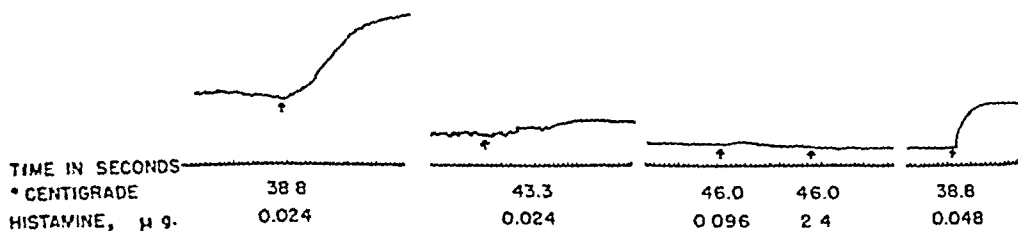
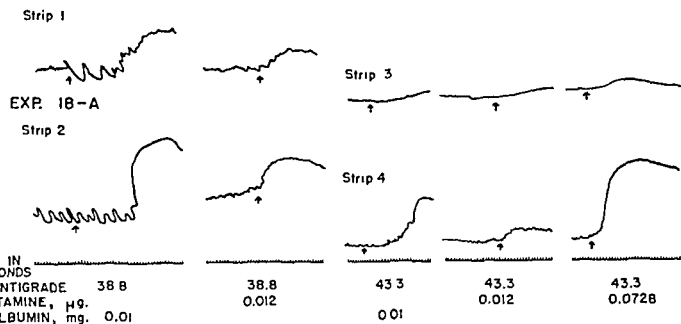


Fig. 2.—The response of ileal strips to histamine at  $38.8$ ,  $43.3$ , and  $46.0^{\circ}\text{C}$ .

at these temperatures. The maximum amount of the stimulant to which the tissues were exposed at these elevated temperatures was  $2.4 \mu\text{g}$  per cubic centimeter. As shown in Fig. 2, there is less contraction to  $2.4 \mu\text{g}$  per cubic centimeter of histamine at  $46.0^\circ \text{C}$ . than to  $0.024 \mu\text{g}$  at  $38.8^\circ \text{C}$ .

*The Response of Sensitized Intestinal Strips Toward the Specific Antigen When Exposed at Various Temperatures.*—Experiments were made with tissue from animals sensitized by intraperitoneal injection of 100 mg. of ovalbumin. Animals weighing approximately 500 grams were used. We were unable to sensitize old guinea pigs to such an extent that a response of the excised ileum to antigen could be provoked consistently when the animals received a single sensitizing dose of ovalbumin. The animals were used from twenty-nine to eighty-six days after the sensitizing injection had been given. The ovalbumin was recrystallized four times according to the method of Cole.<sup>9</sup> Two strips from a 12 cm. segment of ileum were set up in the tissue bath at  $38.8$  or  $39.0^\circ \text{C}$ . and the temperature was slowly raised to  $43.3^\circ \text{C}$ . (from forty to fifty minutes). An amount of ovalbumin was added which, on preliminary test with a trial strip of intestine from the same animal, gave a submaximal contraction. For a con-

## EXP. 18



## EXP. 22

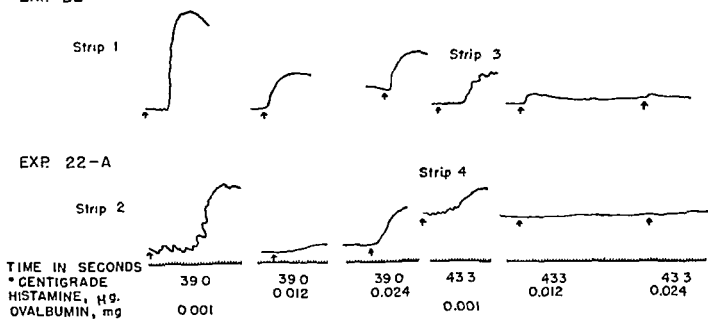


FIG. 3.—The response of sensitized ileal strips to ovalbumin and histamine when exposed to these substances at  $38.8$  to  $39.0^\circ \text{C}$ . and at  $43.3^\circ \text{C}$ .

trol the two remaining strips were kept at 38.8 or 39.0° C. for from forty to fifty minutes. These tissues were exposed to the same amount of ovalbumin as the heated segments. Forty-six of these experiments were made with tissue from nine guinea pigs. In thirty-eight of the tests there was less contraction in the strips heated to 43.3° C. than in the control strips kept at 38.8 or 39.0° C. for from forty to fifty minutes. Eight strips showed equal or greater contraction at the elevated temperature. Four typical tracings are shown in Fig. 3. The mean magnitude of the contractions of the control strips was 17.5 mm. and for the heated strips it was 10.0 mm.

Strips were also heated to 45.0 or 46.0° C. In none of these tests was there any contraction, even when the tissue was exposed to 1.0 mg. per cubic centimeter of antigen. Several typical tracings are shown in Fig. 4. Experiments were then made to determine whether histamine-like substances were released from sensitized ileum at these temperatures. For these tests guinea pigs weighing from 390 to 590 grams were sensitized by a single intraperitoneal injection of 100 mg. of crystallized ovalbumin. From thirteen to thirty-three days later they were killed and approximately 40 cm. of the ileum removed. Detritus within the lumen was washed out with warm Ringer-Locke's solution and the tissue was cut into segments 10 cm. in length. The segments were closed with silk thread. One segment was placed in a culture tube containing 1.0 c.c. of Ringer-Locke's solution previously immersed in a constant temperature bath regulated at 39.0° C. The adjacent segment was also placed in a culture tube containing 1.0 c.c. of Ringer-Locke's solution, but it was immersed in a constant temperature bath regulated at 45.0° C. Three minutes later, after the muscle had attained the temperature of the bath, 1.0 or 10.0 mg. of ovalbumin contained in 1.0 c.c. of Ringer-Locke's solution and having the same temperature as the bath was added to each tissue. The tissues were agitated several times and five minutes after addition of antigen, 1.0 c.c. of liquid was removed from each tube and tested on the excised ileum from normal guinea pigs weighing 300 grams. The strip of normal ileum was suspended in a tissue bath regulated at 39.0° C. which contained 10.0 c.c. of Ringer-Locke's solution. Histamine-like substances could not be detected in all of the tissues tested. Several tracings are shown in Fig. 5. They show that histamine-like substances are released from sensitized ileum at 45.0° C. We were unable to compare accurately the amounts released at 39.0 and 45.0° C. because of the variation in the quantity released by different strips of tissue. Since histamine-like substances are released at 45.0° C., however, it is probable that the effect of heat on sensitized tissue is primarily due to a decrease in the sensitivity of smooth muscle toward the toxic substance released and not to a decrease in the reaction between antigen and fixed antibody.

*The Effect of Fever on the Immediate Type of Skin Reaction.*—Our next concern was whether fever temperatures decreased the sensitivity of sensitized skin tissue as well as of smooth muscle tissue. The size of the wheal in the immediate type of reaction during fever was studied. For these tests guinea pigs weighing approximately 600 grams were used. The reactions produced in this size animal are not as large as the reactions elicited in guinea pigs weighing from 250 to 300 grams, but because such animals are less excitable and therefore easier to handle in the heat cabinet than young animals, they were used. The day before the heat treatment three or four closely clipped animals were injected in the following manner: On both sides of the back and at identical sites, 0.05 c.c. of undiluted antiprotein serum was injected intradermally. The antiprotein serum

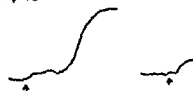
EXP 32

Strip 9

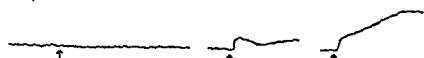


EXP 32-A

Strip 10



Strip 11



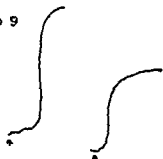
Strip 12



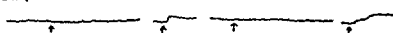
TIME IN SECONDS	38.8	38.8	45.0	45.0	38.8
*CENTIGRADE					
HISTAMINE, $\mu$ g	0.01	0.036		0.036	0.036
OVALBUMIN, mg			1.0		

EXP 24

Strip 9

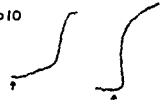


Strip 11



EXP. 24-A

Strip 10



Strip 12

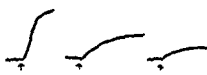


TIME IN SECONDS	39.0	39.0	46.0	46.0	38.8	38.8
*CENTIGRADE						
HISTAMINE, $\mu$ g	0.01	0.024	1.0	0.024	1.0	0.024
OVALBUMIN, mg						

Fig. 4.—The effect of temperatures of 38.8 to 39.0, 45.0, and 46.0° C. on the response to ovalbumin and histamine in sensitized ileal strips.

EXP. 9

EXP. 15



TIME IN SECONDS					
HISTAMINE, $\mu$ g	0.1				0.1
HISTAMINE-LIKE SUBSTANCE	A	B	A	B	

Fig. 5.—The effect of sensitized guinea pig ileum on the release of histamine-like substances from antigen. A, Sensitized ileum and 0.5 mg. of antigen. B, Sensitized ileum and 0.5 mg. of antigen at 38.8° C.

was a pooled serum obtained from young guinea pigs sensitized to horse serum. For a control, 0.05 c.c. of undiluted normal guinea pig serum was injected approximately 3 cm. anterior to, and on either one or the other side of, the back. On the following day 0.05 c.c. of normal horse serum was injected intradermally into one sensitized site and into the control site previously injected with normal guinea pig serum. Sixty minutes later the reactions were measured. The animals which did not react to the injection of normal horse serum at the sensitized site were discarded. One of the animals which showed an immediate type of response to the injection of normal horse serum at the sensitized site was heated at 42.2° C. and the temperature maintained for thirty minutes. The animal was then removed and 0.05 c.c. of normal horse serum was injected into the remaining sensitized site. It was returned to the fever cabinet and the temperature of 42.2° C. maintained for thirty minutes. At the end of this time the cabinet doors were opened and defervescence was allowed to take place. Thirty minutes later, or sixty minutes after injection of antigen, the reaction was measured in two diameters. At approximately the same time that the heat-treated animal was injected with normal horse serum, the remaining control animals that reacted to the antigen were given the same dose of horse serum at the remaining sensitized site. Sixty minutes after injection these reactions were likewise measured in two diameters. The results of these tests are given in Table VII. They show that the wheals formed during hyperpyrexia are significantly smaller than those formed before induction of fever. It is apparent from these results that skin as well as smooth muscle tissue is desensitized during hyperpyrexia.

TABLE VII. COMPARISON OF THE IMMEDIATE TYPE REACTIONS ELICITED BY INTRADERMAL INJECTION OF NORMAL HORSE SERUM IN THE SKIN OF LOCALLY SENSITIZED GUINEA PIGS BEFORE AND DURING A FEVER TEMPERATURE OF 42.2° C.

PIG	FEVERED ANIMALS			PIG	UNFEVERED CONTROL ANIMALS		
	SIZE OF WHEELS ELICITED BY HORSE SERUM IN MM.				SIZE OF WHEELS ELICITED BY HORSE SERUM IN MM.		
	(SENSITIZED SITE) INJECTED BEFORE FEVER	(SENSITIZED SITE) INJECTED 3 TO 4 HOURS AFTER (1) DURING FEVER	(NORMAL SITE) INJECTED BEFORE FEVER		(SENSITIZED SITE)	(SENSITIZED SITE) INJECTED 3 TO 4 HOURS AFTER (1)	(NORMAL SITE)
	(1)	(2)	(3)		(1)	(2)	(3)
C 733	18 by 23	16 by 19	14 by 18	I 643	19 by 21	20 by 22	16 by 20
M 723	24 by 25	16 by 18	19 by 22	I 633	15 by 21	15 by 19	14 by 15
I 650	15 by 18	13 by 15	12 by 12	D 319	16 by 20	21 by 21	13 by 18
J 954	19 by 21	14 by 17	15 by 15	I 171	20 by 22	22 by 23	15 by 17
D 722	17 by 17	12 by 13	14 by 15	I 604	21 by 25	20 by 23	15 by 15
I 466	18 by 20	16 by 19	11 by 16	I 673	20 by 24	20 by 24	13 by 16
DD 573	16 by 21	14 by 17	14 by 16	R 109	15 by 19	15 by 19	14 by 16
V 292	18 by 20	14 by 16	14 by 17	A 749	14 by 18	14 by 18	11 by 16
DD 505	16 by 17	12 by 15	12 by 14	K 736	16 by 18	15 by 18	15 by 16
DD 512	17 by 22	16 by 19	13 by 15	V 282	17 by 19	17 by 19	16 by 17
				V 240	16 by 17	17 by 19	12 by 14
				V 233	15 by 15	17 by 19	12 by 15
				DD 574	16 by 20	17 by 19	15 by 15
				DD 599	13 by 17	18 by 22	11 by 13
Mean di- ameters	17.8 by 20.4	14.3 by 16.8	13.8 by 16.0		16.6 by 19.7	17.7 by 20.4	13.7 by 15.5

#### DISCUSSION

The foregoing experiments indicate that although there is no significant change in the antiprotein titer of an animal's serum during hyperpyrexia, sensitivity to the specific antigen is decreased. All of the heat-treated animals injected with antigen recovered, while six of the eight control animals given the same dose of horse serum died of anaphylactic shock. Since febrile temperatures

had no effect upon the antiprotein content of the serum but did suppress anaphylactic shock, the effect of fever on histamine intoxication was studied to ascertain whether hyperpyrexia affected sensitivity toward this nonspecific stimulant. The results show that resistance to histamine shock is increased at fever temperatures since 95 per cent of the control animals and only 33 per cent of the heat-treated animals died when given the same dose of histamine. Protection against histamine shock is obtained only during the height of fever. When the temperature has returned to normal after a temperature of  $42.2^{\circ}\text{C}$ . has been maintained for thirty minutes, the animals are as susceptible to histamine as the nonheat-treated control animals. Mirsky and Wasserman<sup>10</sup> also studied the effect of fever on histamine in a series of guinea pigs. They found, however, that fever had no effect on histamine intoxication.

The protection afforded by a fever temperature against histamine is not as great as that afforded against anaphylactic shock. Thirty-five old sensitized guinea pigs, used in these and other experiments not reported here, were heat-treated at a temperature of  $41.7$  or  $42.2^{\circ}\text{C}$ . for periods of thirty or sixty minutes. At the end of the fever period a dose of horse serum was injected. Eighty-eight per cent of the control animals died, while only 3 per cent of the heat-treated animals given the same dose of antigen succumbed. Although it was found difficult to compare the degree of severity in histamine shock with the degree of severity in anaphylactic shock, the symptoms in old heat-treated animals injected with histamine appeared to be more severe than the symptoms in old similarly treated sensitized animals injected with horse serum.

The *in vitro* studies on the surviving intestine exposed to histamine make it appear probable that a decrease in contractility of this tissue during fever is responsible for the protection afforded against this stimulant *in vivo*. The results show that as the temperature is increased from  $38.8$  to  $46.0^{\circ}\text{C}$ ., contractility of excised smooth muscle, when exposed to histamine, decreases. When the same tissue was tested at  $38.8^{\circ}\text{C}$ ., at  $43.3^{\circ}\text{C}$ ., and again, after cooling, at  $38.8^{\circ}\text{C}$ ., approximately the same response was obtained after the last as after the first exposure to histamine. This appears to indicate that there is little tissue damage at  $43.3^{\circ}\text{C}$ . This was not the case after exposure to temperatures of  $45$  or  $46^{\circ}\text{C}$ ., however, since there was always less response to histamine after the tissue was cooled at  $38.8^{\circ}\text{C}$ .

In thirty-eight of forty-six tests, intestinal strips from animals sensitized to ovalbumin likewise showed less response at  $43.3^{\circ}\text{C}$ . than at  $38.8^{\circ}\text{C}$ . The eight failures may have been due to the fact that ileal strips from the same animal are not exactly identical in their sensitivity. Slight differences in the length of the tissue, for which we did not attempt to correct, may also play a role. In the experiments conducted at  $45$  and  $46^{\circ}\text{C}$ . there was marked loss of response on exposure to ovalbumin. These *in vitro* experiments indicate that a decrease in contractility of smooth muscle tissue at elevated temperatures may be partially responsible for the protection afforded by fever against anaphylactic shock *in vivo*. The greater protection afforded by fever to sensitized guinea pigs injected with an assaulting dose of antigen than to guinea pigs injected with histamine makes it appear probable that still other factors play a role in the desensitization of sensitized guinea pigs by fever temperatures.

#### SUMMARY

An artificially induced fever temperature of  $42.2^{\circ}\text{C}$ . maintained for thirty minutes has no effect upon the humoral precipitin titer of guinea pigs immunized against horse serum.

The antiprotein titer of sensitized guinea pigs is not significantly altered by a fever temperature of 42.2° C. maintained for sixty minutes when the serum is titrated by a modified Prausnitz-Küstner reaction.

A fever temperature of 42.2° C. maintained for thirty minutes suppresses histamine shock.

In vitro tests with surviving normal intestine exposed to histamine indicate that there is less contraction at 43.3, 45.0, or 46.0° C. than at 38.8 or 39.0° C. Response of sensitized intestine to the specific antigen (ovalbumin) is also decreased by temperatures of 43.3, 45.0, or 46.0° C.

The immediate type of reaction in the guinea pig's skin is suppressed by a fever temperature of 42.2° C. maintained for sixty minutes when the locally sensitized tissue is injected with the specific antigen during hyperpyrexia.

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## STUDIES ON RICKETTSIAL AGGLUTINATION IN TYPHUS

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THE first report of the development of rickettsial agglutinins as the result of infection was made by Otto and Dietrich,<sup>1</sup> who found that the sera of men and rabbits convalescent from typhus agglutinated a rickettsial suspension. Other early workers<sup>2</sup> confirmed this finding, using the same antigen, a suspension prepared from infected lice. In more recent years, with the development of methods for rickettsial multiplication apart from the insect vectors,<sup>3-5</sup> our knowledge concerning agglutinins has been amplified. It is now known that agglutinins develop in man and animals receiving rickettsial vaccines.<sup>6-9</sup> Information with respect to cross-reactions between the murine and epidemic strains, and whether one can distinguish between these infections, is less complete. Using a murine antigen prepared from rats<sup>3</sup> and an infected louse suspension for epidemic antigen, Zinsser and Castañeda<sup>10</sup> showed that sera from human convalescents from both types of typhus gave cross-agglutination. These authors noted that a higher cross-titer was obtained when murine convalescent serum was tested with epidemic antigen than when European serum was set up with murine antigen. Reports on investigations on the absorption of agglutinins from typhus antisera are limited to those of Castañeda and Kligler and their co-workers. It was observed by the former<sup>11</sup> that absorption of typhus serum with a suspension of *Bacillus proteus* removed only the proteus agglutinins, but that if the same serum were absorbed with rickettsial antigen, both proteus and rickettsial agglutinins were removed. Kligler and Oleinik<sup>12</sup> reported that absorption with either epidemic or murine antigen did not remove heterologous agglutinins completely.

The present report deals with the results of agglutination tests obtained over a period of years with a variety of sera from man and animals infected with or vaccinated against typhus, with the cross-agglutinations encountered, and with serum absorption experiments.

### METHODS AND MATERIALS

Some of the agglutinations were performed with the tissue culture antigen previously described,<sup>13</sup> but for the most part the antigens used were prepared from the yolk sacs of infected eggs.<sup>5</sup> Seven-day-old embryos were inoculated with a 1:300 or 1:400 dilution of murine (Wilmington strain) or epidemic (Breinl strain) typhus yolk sac and incubated at 34 to 35° C. The majority of deaths occurred on the sixth and seventh days. Once it became apparent that the chicks were beginning to die, the eggs were candled every two or three hours and the dead ones were kept in the icebox until harvested. The harvested yolk sacs were then shaken by hand in bottles containing glass beads until a smooth suspension was obtained. Physiologic saline containing 0.2 per cent formalin was added to make a 20 per cent suspension and the material was placed at from 2 to 5° C. for from three days to a week, during which time

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it was shaken every day. Microscopic examination revealed that freshly prepared suspensions usually contained cells packed with rickettsiae. In spite of the presence of formalin, autolysis of these cells took place when suspensions were allowed to remain in the icebox, with the consequent liberation of rickettsiae. The suspension was then filtered through gauze to remove any pieces of membrane and extracted in the cold with an equal volume of ether. About eighteen hours later the antigen was drawn off. As a preservative, phenyl mercuric nitrate sufficient to make a final concentration of 1:50,000 was added.

For the agglutination test, glass slides having twelve depressions were used. The serum to be tested was set up in tubes in doubling dilutions and one drop of each dilution was transferred to the slide with a capillary pipette. Using a capillary of the same bore, an equal volume of antigen was added. The slides were then rotated by hand to secure thorough mixing and placed on moist paper in Petri dishes in the incubator at a temperature of 40° C. Although lower temperatures may be employed, agglutination, especially of weakly positive sera, is delayed. For uniform results the slides were left in the incubator for five hours. They were then placed in the icebox overnight, after which a final reading was made with the low-power objective of an ordinary microscope ( $\times 100$ ). Actually, with sera which show a titer of 1:80 or above, agglutination in the lower dilutions is visible to the unaided eye after incubation for from thirty to sixty minutes and in much less time if the rotation of the slide is carried out for two minutes. Where the test is being used for diagnostic purposes, this rapid agglutination of strongly positive sera is of practical importance. Positive and negative serum controls were included on each test. In the absence of agglutination, there is a perfectly smooth distribution of the antigen, which is easily distinguished from even a 1 plus agglutination reading. Any degree of agglutination may be considered significant, since, as will be pointed out, normal sera give no trace of agglutination whatever. Complete agglutination, where the entire field was covered by large clumps, was recorded as a 4 plus reading. In a 3 or 2 plus reaction the clumps were relatively smaller, and there was more space between them. In a reading recorded as a trace or 1 plus, the agglutinated particles were definite but of smaller size than in the case of a 2 plus reaction.

Proteus antigens were prepared fresh as needed from eighteen-hour cultures of *Bacillus proteus* OX19, Lister strain, at a density equal to the No. 3 tube of the McFarland nephelometer. For the agglutination test, 0.5 c.c. portions of antigen suspension were added to equal quantities of the serum dilutions. The tubes were incubated for two hours at 40° C. and placed in the icebox overnight before a final reading was made.

For absorption studies of the sera, 30 per cent antigen was used. Equal parts of antigen were combined with serum diluted 1:2.5 and the mixtures incubated for three hours at 37° C. Following fifteen minutes' centrifugation at 2,000 r.p.m., the supernatant was once more combined with an equal amount of antigen and incubation and centrifugation carried out as before. If necessary this procedure was repeated.

## RESULTS

### *Human Studies.*—

*Typhus Cases and Convalescents:* Hudson<sup>14</sup> found no positive reactions among twenty-five sera from normal adults in Ohio tested against murine typhus antigen. We have never seen typhus agglutinins in sera other than those

of persons with a history of typhus or of vaccination. The sera of seventy-five blood donors from a Philadelphia suburb were completely negative when tested for murine typhus agglutinins. (About 50 per cent had agglutinins for proteus OX19 in dilutions of 1:20 or 1:40.) The sera of thirty-five other normal adults from the Philadelphia area showed no agglutinins for epidemic antigen. By contrast, among 155 sera\* from persons in Alabama with a clinical history of typhus within two years before bleeding, 104 gave positive results with murine antigen. It is possible that those giving negative results did not have typhus or that their agglutinins had disappeared. Among the positives, titers varied from 1:20 to 1:1280. In fifty-eight of the 104 positives, proteus agglutinins were absent or lower than those for typhus, showing a tendency in man, previously observed in rabbits,<sup>15</sup> toward a persistence of rickettsial agglutinins. In eighteen of the 104 patients the proteus agglutinins were higher than those for murine antigen. In some cases, no doubt, this might be a manifestation of an increase of proteus agglutinins normally present. It may be noted here that a pool of twenty of the sera showing the highest murine rickettsial titer gave no agglutination with spotted fever antigen.

Two cases of suspected epidemic typhus in persons exposed to infection during the production of typhus vaccine<sup>16</sup> were diagnosed beyond doubt by the increase in titer of the rickettsial agglutinins above the level induced by vaccination. This rise in titer occurred in one case on the fifth day of fever, and in the other on the eighth day of illness when the temperature had been normal for two days. Heterologous agglutinins (murine typhus) were also present, as was to be expected, but in lower titer than that of the infecting strain. The serum studies on these two cases are summarized in Tables I and II.

TABLE I. AGGLUTINATION TESTS ON SAMPLES OF SERUM FROM CASE 1  
(EPIDEMIC TYPHUS)

SERUM SAMPLE	DILUTION OF SERUM												
	PROTEUS OX19					EPIDEMIC ANTIGEN				MURINE ANTIGEN			
	20	40	80	160	320	20	40	80	160	20	40	80	160
Before vaccination	3*	2	1	±	-	0	0	0	0	-	-	-	-
After 3 c.c. vaccine†	3	2	1	±	-	1	±	0	0	±	0	0	0
5th day of fever‡	3	3	2	1	0	2	2	1	0	1	±	0	0
6th day of fever	3	3	2	1	0	3	2	1	0	1	±	0	0
13th day after onset	4	4	4	3	3	3	3	3	1	3	3	2	2
31st day after onset	4	4	3	2	±	4	4	3	2	4	3	2	1
54th day after onset	4	4	2	2	±	4	4	3	1	3	2	1	±
109th day after onset	3	2	1	0	0	3	2	2	1	2	1	±	0

\*± denotes trace of agglutination; 1, 2, 3, 4, degree of agglutination; 0, negative, - not done.

† Bleeding taken ten days after third dose.

‡ Blood infectious for guinea pigs on this day (two months after vaccination).

TABLE II. AGGLUTINATION TESTS ON SAMPLES OF SERUM FROM CASE 2  
(EPIDEMIC TYPHUS)

SERUM SAMPLE	DILUTION OF SERUM													
	PROTEUS OX19				EPIDEMIC ANTIGEN						MURINE ANTIGEN			
	20	40	80	160	20	40	80	160	320	640	20	40	80	160
Before vaccination	2	1	0	0	0	0	0	0	-	-	0	0	0	0
After 4.5 c.c. vaccine*	2	1	0	0	2	1	1	0	0	0	2	1	0	0
1st day of fever†	2	1	0	0	2	0	0	0	0	0	-	-	-	-
6th day after onset‡	2	1	0	0	2	0	0	0	-	-	-	-	-	-
8th day after onset	3	2	1	±	3	3	2	2	2	1	4	3	2	1
11th day after onset	3	2	1	±	4	3	3	3	3	2	4	3	3	2
70th day after onset	2	1	0	0	4	4	3	2	1	0	4	3	2	1

\*Bleeding was taken five weeks after fifth dose.

†Ten weeks after vaccination.

‡Blood not infectious for guinea pigs on this day.

\*These sera were obtained for us by Dr Samuel R. Damon, of the State Board of Health Laboratories, Montgomery, Ala., to whom we express our thanks.

*Vaccinated Subjects:* During the course of epidemic typhus vaccine production in these laboratories, more than ninety persons were vaccinated. Some received three 1 c.c. doses of centrifuged yolk sac vaccine<sup>16</sup> which, on smear, showed very few rickettsiae. The others were given three or five 1 c.c. doses of ether-extracted vaccine made from rich yolk sac material. Samples of serum\* were obtained from thirty-five of the subjects before vaccination, but this step was later omitted due to the pressure of other work. Serum was collected ten days after the last dose in most instances. The agglutinin responses for proteus OX19 and for epidemic typhus rickettsiae were studied. Space does not permit the tabulation of results, but the following facts were brought out in this study: (1) that regardless of the type of vaccine used, or the amount, there was considerable variation in individual response; (2) that the more potent vaccine in most cases gave higher agglutinin titers; (3) that negative rickettsial agglutination was found in only one instance; a poor response (1 plus agglutination at a 1:20 dilution of serum only) in six additional cases. These subjects were all in the group that had received the vaccine which, on smear, showed few rickettsiae. In those instances where blood was obtained after the third and again after the fifth dose, there was usually found to be no increase in titer at the second bleeding. In the cases where a comparison could be made with the prevaccination bleeding, the proteus agglutinins elicited by vaccination were not significant.

*Animal Studies.*—The formation of rickettsial agglutinins as the result of infection has been reported for rabbits,<sup>1, 15</sup> guinea pigs,<sup>10</sup> and monkeys.<sup>7</sup> Except in the case of rabbits, no observations have been reported on the time of appearance and the titers obtained in animals. It was felt, therefore, that pertinent information might be revealed by a study of the agglutinin response of animals following infection and vaccination and that the results might have practical application. Guinea pigs, mice, wild rats, and hamsters were studied.

*Wild Rats:* Philip and Parker<sup>18</sup> reported that in the case of laboratory rats the brain was infectious for 370 days. Unfortunately, the duration of agglutinins in this animal has not been determined and it is not known whether or not this antibody outlasts the period of infectivity of the rat brain.

Twenty-three sera from infected rats caught in Savannah† were set up with proteus OX19 and with murine and epidemic typhus antigens. Fifteen of the sera were positive for rickettsial agglutinins; only one had clear-cut proteus agglutinins. It was also found that where agglutinins for murine antigen were present there was usually agglutination of the heterologous suspension as well, although always in lower titer.

*Guinea Pigs:* In guinea pigs, considerable variation was encountered. In a group of animals receiving the same infectious material or vaccine the agglutinin response varied widely. The individual responses of guinea pigs inoculated with epidemic passage brain and vaccine, respectively, are recorded in Table III.

In guinea pigs infected with epidemic typhus, the peak of agglutinins was reached about ten days after defervescence. In animals bled on the fifth or sixth day of fever (eleventh or twelfth day after inoculation) no agglutinins were detectable.

In vaccinated pigs the appearance of agglutinins was also delayed. Titers were low and dropped rather rapidly. Better agglutination was found two weeks after vaccination than after three weeks.

\*We are indebted to Dr. Leroy Wenger for his cooperation in performing these venipunctures.

†These were obtained by Dr. George Brigham, of the U. S. Public Health Service, and kindly forwarded to us by Dr. Ida Bengtson, of the National Institute of Health.

TABLE III. AGGLUTININS IN THE SERA OF GUINEA PIGS INJECTED WITH EPIDEMIC TYPHUS VACCINE OR BRAIN SUSPENSION\*

GUINEA PIG	INOCULUM††	DILUTION OF SERUM			
		4	8	16	32
1	Vaccine	3	2	0	0
2	Vaccine	4	3	2	0
3	Vaccine	2	1	0	0
4	Vaccine	3	3	2	0
5	Vaccine	2	1	0	0
6	Vaccine	2	1	±	0
		DILUTION OF SERUM			
		20	40	80	160
1	Infected brain	2	±	0	0
2	Infected brain	4	3	3	3
3	Infected brain	4	3	3	2
4	Infected brain	4	4	3	3
5	Infected brain	3	3	2	1
6	Infected brain	4	3	2	1

\*Blood taken two weeks after inoculation

†Animals received two 0.5 c.c. doses of vaccine.

††Animals received 1 c.c. 5 per cent brain suspension.

In guinea pigs infected with murine typhus (guinea pig tunica suspension) agglutinins developed much earlier than in pigs inoculated with brain suspension of the epidemic strain, but the peak was reached about the same time in both cases. It remained to be shown whether this was a strain characteristic or was due to the greater number of rickettsiae present in the endemic passage inoculum. Two guinea pigs each were infected with a 1:100 dilution of comparable yolk sacks of the two strains and bled on the fourth and sixth days of fever and on the fourteenth day after inoculation. As is seen in Table IV, there was no strain difference as to the formation of agglutinins under these conditions, and for both types of typhus titers were higher than usually found when guinea pig passage material was used as infectious inoculum.

TABLE IV. AGGLUTINATION TESTS ON THE SERA OF GUINEA PIGS INFECTED WITH 1 C.C. OF A 1:100 DILUTION OF MURINE OR EPIDEMIC YOLK SAC SUSPENSION\*

GUINEA PIG	STATUS	ANTIGEN	DILUTION OF SERUM			
			20	40	80	160
M. T. †1	4th day of fever	Murine	4	4	4	3
M. T. 2	4th day of fever	Murine	4	4	3	2
M. T. 1	6th day of fever	Murine	4	4	4	3
M. T. 2	6th day of fever	Murine	4	4	4	3
M. T. 1	14th day of disease	Murine	3	4	4	4
M. T. 2	14th day of disease	Murine	4	4	4	3
E. T. †1	4th day of fever	Epidemic	4	4	3	2
E. T. 2	4th day of fever	Epidemic	4	4	4	3
E. T. 1	6th day of fever	Epidemic	4	4	3	2
E. T. 2	6th day of fever	Epidemic	4	4	4	3
E. T. 1	14th day of disease	Epidemic	4	4	4	4
E. T. 2	14th day of disease	Epidemic	4	4	4	4

\*It may be noted here that small amounts of precipitins to yolk sac may be formed in guinea pigs, but the reactions are weak and irregular and do not obscure the results obtained in an experiment such as the above. A 1 plus at the 1:20 dilution of serum was the highest titer found in these guinea pigs with normal yolk sac antigen.

†M. T. denotes pigs infected with murine yolk sac.

†E. T. denotes pigs infected with epidemic yolk sac.

*Mice:* Experiments with mice (Swiss, albino) showed these animals to be very satisfactory for agglutinin study. Sera from fourteen normal mice had no agglutinins for either typhus antigen. Agglutinins were found to be present as early as five days after intravenous inoculation of these animals with 0.5 c.c. of epidemic typhus yolk sac suspension at a 1:400 dilution. By using murine typhus yolk sac as inoculum and injecting by the intraperitoneal route with 0.5 c.c. of a 1:400 dilution, it was possible to detect agglutinins on the fifth,

but not on the fourth, day. By the seventh day much higher titers were found with both strains (Table V). Vaccination of mice with vaccines of both types of typhus<sup>19</sup> produced in each case good agglutinin response by the seventh day, although, as might be expected, titers were not as high as when living rickettsiae were injected.

TABLE V. AGGLUTININS IN THE SERA OF MICE RECEIVING TYPHUS YOLK SAC\*

MOUSE	INFECTING STRAIN	ROUTE†	DILUTION OF SERUM			
			20	40	80	160
1	Epidemic	i.v.	3	2	1	0
2	Epidemic	i.v.	4	3	2	1
3	Epidemic	i.v.	4	3	3	2
4	Murine	i.p.	4	3	2	2
5	Murine	i.p.	4	3	2	0
6	Murine	i.p.	3	2	1	0

\*Blood obtained one week after the inoculation of 1:400 yolk sac suspension.

†I.V. Intravenously; I.P. intraperitoneally.

*Hamsters:* Hamsters were found to be very resistant to inoculation with epidemic typhus.<sup>20</sup> The intraperitoneal injection of 1 c.c. of a 10 per cent yolk sac suspension produced no febrile reaction and very feeble rickettsial agglutinins when tests were made eight days after inoculation. Because of their lack of susceptibility to infection, further work with these animals was not undertaken.

*Cross-Agglutination.*—In all of the species studied, sera from animals inoculated with infectious material gave agglutination with the heterologous typhus antigen with but one exception. This occurred in the case of mice infected intravenously with epidemic typhus (*vide infra*).

TABLE VI. HOMOLOGOUS AND HETEROLOGOUS AGGLUTINATION TESTS ON HUMAN SERA FROM CASES OF MURINE TYPHUS\*

CASE	ANTIGEN	DILUTION OF SERUM							
		20	40	80	160	320	640	1280	2560
1	Murine	4	4	4	4	3	2	1	±
1	Epidemic	3	3	2	2	1	0	0	0
2	Murine	4	4	4	4	3	2	0	0
2	Epidemic	4	3	2	1	0	0	0	0
3	Murine	4	4	4	4	3	3	2	1
3	Epidemic	4	4	3	2	2	1	±	0

\*The sera of these patients and twenty other convalescent murine typhus patients tested showed no agglutination with spotted fever antigen.

Examples of cross-agglutination in human cases of epidemic typhus were given in Tables I and II. In human sera from the endemic disease, the heterologous agglutinins were likewise present but again not in sufficiently high titer to confuse the diagnosis. The reactions observed with sera from three patients with murine typhus, using both types of antigen, are shown in Table VI.

In the case of guinea pigs, cross-agglutination was evident once the homologous antibody was present in more than minimal titer. This is illustrated in Table VII, which shows the cross-agglutination found for passage pigs that had received either epidemic brain suspension or murine tunica.

TABLE VII. CROSS-AGGLUTINATION IN THE SERA OF GUINEA PIGS

GUINEA PIG	INFECTING STRAIN	ANTIGEN	DILUTION OF SERUM					
			20	40	80	160	320	640
1	Epidemic	Epidemic	4	4	4	4	3	2
1	Epidemic	Murine	3	2	2	1	0	0
2	Murine	Murine	4	4	4	3	2	2
2	Murine	Epidemic	3	2	0	0	0	0

Cross-agglutination was found in the sera of wild rats as mentioned previously. It did not occur where the homologous antibody had dropped to a low titer.

TABLE VIII. CROSS-AGGLUTINATION IN THE SERA OF MICE RECEIVING MURINE OR EPIDEMIC INFECTIOUS YOLK SAC\*

MOUSE	INFECTING STRAIN	ROUTE†	DILUTION OF SERUM							
			MURINE ANTIGEN				EPIDEMIC ANTIGEN			
			20	40	80	160	20	40	80	160
1	Murine	I.P.	4	3	2	2	4	3	2	1
2	Murine	I.P.	4	3	2	0	3	2	1	0
3	Murine	I.P.	3	2	1	0	2	0	0	0
4	Murine	I.P.	2	1	±	0	2	±	0	0
5	Murine	I.P.	3	2	1	0	3	1	±	0
6	Murine	I.P.	4	3	2	1	3	2	1	0
1	Epidemic	I.V.	0	0	0	0	3	3	2	1
2	Epidemic	I.V.	0	0	0	0	3	2	2	1
3	Epidemic	I.V.	0	0	0	0	3	2	1	0
4	Epidemic	I.V.	±	0	0	0	4	3	3	2
5	Epidemic	I.V.	±	0	0	0	4	3	3	2
6	Epidemic	I.V.	±	0	0	0	4	3	2	1

\*Blood obtained one week after inoculation of 1:400 yolk sac suspension.

†I.P. Intraperitoneally; I.V. intravenously.

In mice inoculated intraperitoneally with virulent murine yolk sac material diluted from 1:400 to 1:1000, heterologous agglutinins were present by the seventh day, at which time the homologous titer was usually high (Table VIII). In mice infected by the intravenous route with epidemic typhus yolk sac at a 1:400 dilution, a good titer of homologous agglutinins was likewise in evidence by the seventh day, but no cross-agglutination occurred (Table VIII). The result was the same for all mice infected with epidemic typhus intravenously; cross-agglutination was almost entirely negative. This conceivably might be due to strain difference or might be a function of the route of administration. To determine which of these hypotheses was correct, groups of mice were injected with epidemic infectious material, using the intraperitoneal route. Some cross-agglutinins resulted from this treatment but they did not equal the titers found in mice given murine typhus material by the same route (Table VIII), so that the phenomenon must at least in part be due to a strain difference as regards the mouse. Smears made at autopsy on numerous mice as early as two and one-half hours after intravenous inoculation with epidemic yolk sac material disclosed that the rickettsiae had disappeared. On rare occasions a very few organisms were found in smears made from spleen and blood.

*Agglutinin Absorption Tests.*—Agglutinins were readily absorbed from immune serum. With a 30 per cent rickettsial suspension, it was found that two or three absorptions (depending on the original titer of the serum) usually would remove all of the agglutinins completely for the antigen used, leaving the heterologous antibody intact. The results obtained when murine convales-

TABLE IX. AGGLUTININ ABSORPTION OF MURINE GUINEA PIG SERUM WITH EPIDEMIC ANTIGEN

	DILUTION OF SERUM			
	20	40	80	160
Murine serum + Murine antigen before absorption	4	4	3	2
Murine serum + Epidemic antigen before absorption	4	3	2	1
Murine serum + Murine antigen after 1st absorption	4	4	3	2
Murine serum + Epidemic antigen after 1st absorption	3	2	1	0
Murine serum + Murine antigen after 2nd absorption	4	4	3	2
Murine serum + Epidemic antigen after 2nd absorption	±	0	0	0
Murine serum + Murine antigen after 3rd absorption	-	4	3	2
Murine serum + Epidemic antigen after 3rd absorption	-	0	0	0

cent passage guinea pig serum was absorbed with the heterologous antigen are shown in Table IX. With sera from human patients with endemic typhus, it was found that absorption with proteus antigen left the rickettsial agglutinins intact even after six absorptions. Absorption of the same sera with murine rickettsial antigen removed both proteus and rickettsial antibodies.

#### DISCUSSION

The results of the serologic tests in both man and animals indicate that the rickettsial slide agglutination test using a concentrated antigen may be a useful diagnostic aid in studies on typhus. To be of use, however, a diagnostic agent must be shown to be specific and to give negative results with known normal sera.

With the test as performed in the foregoing experiments, no agglutination with either typhus antigen was encountered in numerous samples of normal serum from man and animals. In addition, sera from twenty cases of murine typhus tested singly and twenty others with high murine titers, tested as a pool, gave no agglutination with spotted fever antigen. Furthermore, we have found no agglutinins for typhus antigen in fifteen pools of sera from rabbits hyperimmunized with large quantities of live spotted fever rickettsiae, each pool containing the sera of from fourteen to eighty-two rabbits. The antigens employed may therefore be considered to have fulfilled accepted criteria for specificity.

The specificity of the rickettsial agglutination test has been seriously questioned by Plotz and co-workers<sup>21</sup> in a recent report, in which sera from thirteen of fourteen cases diagnosed as spotted fever were found to give agglutination with typhus antigens, using a macroscopic tube agglutination technique. The details of the technique used were not described and the results with spotted fever antigen were not given. This report is at variance not only with the work described in this paper where the test was found to be specific, but also with unpublished observations in this laboratory on the sera of spotted fever cases, and is therefore puzzling.

The usual procedure for the detection of typhus in a rat population (i.e., the inoculation of rat brains into guinea pigs) is tedious and expensive. Recently, Brigham and Bengtson<sup>22</sup> have suggested the use of the complement fixation test to detect murine typhus in rats as an aid in rat control measures. The results of our studies on the human and rat sera from two of our southern states suggest that city or county surveys for the presence of typhus could be made equally as well by the simple means of the agglutination test.

In mild cases of human typhus, the agglutination test has been found particularly useful. Here the clinical diagnosis is difficult and the isolation of typhus rickettsiae from the patient's blood is a lengthy procedure which may give negative results. Even when positive results are obtained, guinea pigs must be reinfected with known typhus material to prove their original infection to have been due to typhus, and the long-delayed diagnosis becomes one of academic interest only. The agglutination test, requiring but one reagent, offers a simple and rapid method for early diagnosis and for the differentiation of strains of this infection from each other and from spotted fever. In investigative work also, the agglutination test has been found useful for the diagnosis of mild or inapparent typhus infections in the guinea pig, particularly in the epidemic variety of the disease. In this infection, unlike murine typhus and spotted fever in guinea pigs, no serotal involvement occurs to guide the investigator in the interpretation of a febrile state and many experiments must be repeated because of concurrent infection.

The amount of cross-agglutination occurring in sera has been found to depend on the height of the homologous titer in the serum being studied. When serum dilutions were carried out to the end point, there was usually no difficulty in deciding which strain of typhus was the cause of the infection. Generally speaking, the murine sera gave better cross-agglutination than did the epidemic sera.

The results of absorption tests confirm Castañeda and Zia's<sup>11</sup> observation. The absorption experiments were clear cut, and removal of heterologous agglutinins was complete providing that absorption was carried out a sufficient number of times.

The test, though time consuming, may be used with relative ease due to the availability of rich yields of rickettsiae in yolk sacs and may be employed to advantage in the rare cases where the titers of the patient's serum are almost the same for both antigens.

#### SUMMARY

The microscopic agglutination test as used was found to be an easy and convenient means for the diagnosis of typhus in human sera and proved to be a useful tool for the study of agglutinin development in man and animals following infection or vaccination. The method has been found to be simple and specific. Cross-agglutination reactions are discussed. The observations of Castañeda on the removal of heterologous typhus agglutinins were confirmed. Sera from rabbits infected with spotted fever did not agglutinate either of the typhus antigens. Sera obtained from cases of endemic typhus were found not to agglutinate spotted fever antigen. The procedures for the preparation of the antigens and for carrying out the tests are described.

Since this manuscript was completed, results of agglutination tests using a mouse lung antigen have been published (Castañeda, M.: *J. Immunol.* 50: 179, 1945) which corroborate the findings in this paper.

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## THE BEHAVIOR OF BLOOD CHOLESTEROL IN THYROTOXIC PATIENTS UNDER TREATMENT WITH THIOURACIL

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THE influence of thiourea derivatives upon the blood cholesterol of thyrotoxic patients has been previously mentioned by two groups of observers.<sup>1, 2</sup> Jennings and his associates noted a rapid rise of the blood cholesterol in twelve patients who were taking thiourea. Stable levels were rapidly attained, from which only minor subsequent variations were observed. In their hands, thiouracil administered to a single patient for four months exerted a similar action upon the blood cholesterol.

We have estimated blood cholesterol in the fasting specimens of fifty-two of seventy-eight thyrotoxic patients and have made serial analyses in thirty-six of these. None of the thirty-six had a co-associated diabetes mellitus. Total cholesterol was determined by a method previously described by one of us (I. J. D.<sup>3</sup>). The normal range of values by this method is from 150 to 200 mg. per 100 c.c. of plasma. The free fraction of cholesterol was estimated by the Shoenheimer-Sperry technique<sup>4</sup> modified so as to require but 0.5 c.c. of serum for each analysis. All of these patients had clinical manifestations clearly confirming the toxic status of the thyroid.

The results of our study are summarized in Table I and Fig. 1.<sup>5</sup> No actual data for the free fraction of the blood cholesterol are included as this always varied directly with the total and represented from 25 to 35 per cent of that figure in any individual instance. In eleven of the fifty-two individuals the initial level for total cholesterol in the blood was above 200 mg. per 100 c.c. The averaged mean pretreatment basal metabolic rate for these eleven was +48.5 per cent with a low figure of +37 and a high of +64 per cent, respectively. These facts show that thyrotoxicosis may and does occur in the presence of

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hypercholesterolemia. Therefore, the fasting blood level of this substance cannot be relied upon as an infallible index for either the diagnosis of thyrotoxicosis or its treatment.

TABLE I. RELATION OF ABSOLUTE VALUES FOR CHOLESTEROL (MG. PER 100 C.C. OF BLOOD) TO THE BASAL METABOLIC RATE IN FIFTY TWO PATIENTS WITH THYROTOXICOSIS UNDER TREATMENT WITH THIOURACIL

	NUMBER OF CASES	BASAL METABOLIC RATE		
		15	BETWEEN 0 AND 10	BETWEEN 10 AND 15
Pretreatment	52			
Values above 200	11	11	--	--
After treatment	36			
Values above 200	26		14	12

However, in any single thyrotoxic individual, it has been repeatedly demonstrated that the blood level for cholesterol rises from its initial point as the basal metabolic rate falls (Fig. 1). If absolute values are used, it is impossible to plot an informative curve because of the wide variation from patient to patient in the actual concentrations of cholesterol in the blood. For instance, the lowest pretreatment value for total cholesterol was 91 mg. per 100 c.c. in a patient whose corresponding basal metabolic rate was +65 per cent; the highest pretreatment blood level for the same substance was 374 mg. per 100 c.c. in a nondiabetic patient with a corresponding basal metabolic rate of +42 per

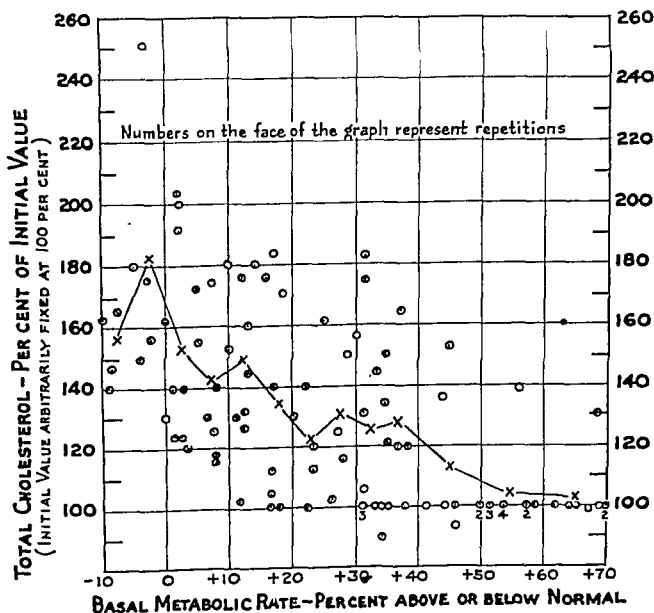


Fig. 1.—Percentual changes in cholesterol in relation to alterations in the basal metabolic rates of patients with thyrotoxicosis under thiouracil treatment (serial examinations in thirty-one patients).

cent. Of the fifty-two patients whose blood levels for cholesterol were studied prior to receiving thiouracil, four showed less than 100 mg. per 100 c.c.; eleven more than 200 mg. per 100 c.c. The initial level for total cholesterol in the blood lay between 100 and 149 mg. per 100 c.c. in sixteen patients and between 150 and 199 mg. per 100 c.c. in twenty-one patients.

Of the thirty-six fully controlled patients whose total blood cholesterol was estimated, four had post-treatment values between 150 and 199 mg. per 100 c.c. In twelve, the figures lay between 200 and 249 mg. per 100 c.c.; in thirteen, between 250 and 299 mg. per 100 c.c.; in five, between 300 and 389 mg. per 100 c.c.; and in two, were over 400 mg. per 100 c.c.

It is obvious from these data that no satisfactory curve could possibly be plotted from the absolute figures for the total cholesterol in the blood. However, when we arbitrarily fix the initial or pretreatment figure at 100 per cent and plot all others percentually therefrom, the "scattering" is not so great and the trend of the mean value for the blood cholesterol in all cases moves inversely with the basal metabolic rate (Fig. 1). A sufficient number of determinations have not been made to establish a mathematical curve with certainty, but a straight line relationship seems likely at least within values for the basal metabolic rate of from  $-10$  to  $+70$ .

The averaged initial value for all determinations in fifty-two individuals was 170 mg. per 100 c.c. of blood; the averaged maximum value in thirty-six subjects, during or after treatment, was 245 mg. per 100 c.c. The mean basal metabolic rates corresponding to these two determinations were  $+48.8$  and  $+11.6$ , respectively. From Table I it will be further noted that the blood cholesterol was high in 33.3 per cent of the patients when their basal metabolic rates lay between  $+10$  and  $+15$ , while a second third (actually 38.8 per cent) developed values above 200 mg. per 100 c.c. when the metabolic rates fell between 0 and  $+10$ .

Such high values for cholesterol in the blood in the presence of normal or slightly elevated basal metabolic rates may indicate some aberration of fat metabolism or transport in the individual whose thyrotoxicosis has been relieved by thiouracil. It is our experience that these patients are clinically at their best when their basal metabolic rates range from  $+5$  to  $+15$ .<sup>2, 5</sup> This conforms to the zone at which high cholesterol values begin to make themselves evident, an academically interesting fact with important obvious clinical implications.

Other practical applications of the blood cholesterol determination in the thiouracil-treated thyrotoxic patient are not wanting. In patients who suffer from claustrophobia or who for some other reason fear the taking of a basal metabolic rate, careful attention to the values for blood cholesterol may enable the clinician to obtain optimum results from therapy with thiouracil. For instance, successive basal metabolic rates at weekly intervals in a highly nervous, easily excitable patient (No. 31)<sup>5</sup> were  $+87$ ,  $+76$ ,  $+81$  and  $+74$ . During this time there was a continual steady improvement in the clinical picture, and the values for blood cholesterol corresponding to the above metabolic rates were 112, 121, 183, and 240, respectively. Operation for nodular goiter was performed without difficulty and the postoperative hospitalization was short and uneventful.

#### SUMMARY AND CONCLUSIONS

1. Fasting blood cholesterol values above 200 mg. per 100 c.c. have been obtained in eleven of fifty-two patients with untreated thyrotoxicosis.

2. In thirty-six of these, serial examinations during treatment revealed a more or less straight-line relationship between the percentual elevation of the blood cholesterol and the falling basal metabolic rate.

3. Under treatment, high values for blood cholesterol were obtained in the majority of patients whose basal metabolic rates had been reduced to +10 or below, and in a considerable number in whom the rate lay between +10 and +15. Clinically, patients felt "at their best" when the basal metabolic rates lay between +5 and +15 and had concomitantly high normal values for blood cholesterol.

4. It is suggested that either the basal metabolic rate or the cholesterol value or a combination of the two may be used as a guide for the satisfactory management of thyrotoxicosis with the drug thiouracil.

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#### A NOTE ON THE IN VITRO RESPIRATION OF MUSCLE IN MYASTHENIA GRAVIS

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EXPERIMENTS previously reported<sup>1</sup> have suggested that the in vitro respiration of skeletal muscle from patients with myasthenia gravis may show certain abnormalities. The present paper describes a continuation of that type of investigation and is a report of experiments on the respiration of skeletal muscle tissue from two additional patients with this disease.

#### EXPERIMENTAL

The experiments consist of measurements of oxygen uptake of respiring muscle obtained at biopsy, and the effect of various supplements—dicarboxylic acids, cytochrome C, prostigmine, ephedrine, cocarboxylase, insulin, and "muscle juice"—on the respiration of the minced muscle. The details of technique have been described.<sup>2</sup> Dicarboxylic acid supplements were in final concentration of 0.003 M. The cytochrome was a purified sample of cytochrome C prepared according to the method of Keilin and Hartree<sup>3</sup>; 100 micrograms were used in each of the flasks that contained cytochrome. The concentrations of other supple-

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These studies were made in 1940 and publication deferred in the hope that additional observations would be made. Circumstances have prevented any further experimentation and it is thought desirable to publish them at this time, incomplete as they are.

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ments per vessel were as follows: prostigmine bromide, 0.5 mg.; ephedrine sulfate, 0.5 mg.; cocarboxylase, 100 micrograms; insulin, 4 units; and muscle juice, 0.2 c.c. The muscle juice was prepared as previously described.<sup>4</sup> The total volume of the contents of the vessels was 2.8 c.c. All results are reported in cubic millimeters of O<sub>2</sub> per milligram of dry weight of tissue.

### CASE HISTORIES AND RESULTS

CASE 1.—H. E., a woman physician, aged 48 years, had first experienced symptoms of myasthenia gravis twenty-one years previously. Intermittent diplopia was noticed five years later, and this was followed in the next year by weakness of the arms and legs and of other muscles innervated by the cranial nerves. The symptoms became worse following each of several upper respiratory infections. Her case was intensively studied at the Presbyterian, Michael Reese, and Billings Hospitals in Chicago, at the University of Minnesota Hospital, and at the Mayo Clinic. The diagnosis of myasthenia gravis was confirmed in each institution and various therapies were tried without success. For nearly six years she was bedridden and unable even to turn over. In 1930 she published her accidental discovery that ephedrine produced remarkable alleviation of her symptoms.<sup>5</sup> Using this drug she improved to such an extent that she was able to travel around the world. In recent years she has been almost normally active while taking 15 mg. of prostigmine bromide from four to six times daily, supplemented by ephedrine sulfate in doses of from 25 to 50 mg. daily.

The patient entered Billings Hospital to submit to a muscle biopsy. Her last dose of prostigmine (15 mg.) had been taken the evening of entry and she received no more until after the biopsy twenty-five hours later. The morning after admission she appeared in good health and normally active, her only difficulty being, as usual, in combing her hair. Her height was 168 cm.; weight, 74.3 kilograms; temperature, 98.6° F.; and pulse, 87. The muscles of the limbs were flabby and became fatigued on repeated movements. A slight bilateral ptosis of the eyelids was present but there was no wrinkling of the forehead. The eyes moved normally. Swallowing and speaking were well performed. There were no significant findings in the lungs, heart, or abdomen. The blood pressure was 118/78. All tendon reflexes were slightly hypoactive. The blood showed no abnormality except a mild hypochromic anemia. The urine was normal.

The day after admission the patient received no medication. In the afternoon she felt weak and very tired and ptosis of the lids was observed. She had some difficulty in helping herself onto the cart which took her to the operating room in the late afternoon where about 8 Gm. of fat-free muscle were removed from the gastrocnemius under local anesthesia. Great care was taken to avoid infiltration of the muscle itself, excision of which was therefore momentarily painful. The tissue was immediately placed in an ice-cold container and taken to the laboratory. On return to her room the patient received 30 mg. of prostigmine orally. Convalescence was uneventful.

TABLE I. THE EFFECT OF VARIOUS SUPPLEMENTS ON THE RESPIRATION OF MUSCLE IN MYASTHENIA GRAVIS—CASE 1 (H. E.)

SUPPLEMENTS	OXYGEN UPTAKE (TOTAL UO <sub>2</sub> VALUES)			
	1ST HR.	2ND HR.	3RD HR.	4TH HR.
0.01 M glucose in buffer				
O—Control (Values are average of duplicate runs)	1.4	1.9	2.2	2.3
Succinate	2.5	5.2	7.8	9.0
Succinate + cytochrome	4.2	7.5	10.6	13.0
Cytochrome	2.4	4.9	7.3	8.7
Prostigmine	2.6	5.0	7.3	8.4
Succinate + prostigmine	3.7	6.8	9.4	11.7
Fumarate	2.6	5.4	8.2	10.5
Citrate	2.4	4.5	6.5	8.2
Glutamate	2.8	5.3	7.8	10.2
Muscle juice	3.0	5.8	8.6	10.2
Succinate + muscle juice	3.8	6.2	8.3	10.2
Succinate + cytochrome + prostigmine + cocarboxylase + insulin + muscle juice	4.5	8.2	11.9	15.0
As directly above but with no succinate	2.9	5.4	7.8	9.5
Succinate + cytochrome + prostigmine	4.0	7.3	10.4	13.0
Cytochrome + prostigmine	2.5	4.8	7.1	8.8

Results of the experiments on the respiration of this tissue are given in Table I and Fig. 1. The latter also shows for comparison results of a similar experiment with "normal human muscle" represented by a biopsy of the abdominal rectus removed during a cholecystectomy using spinal anesthesia. It is observed that the oxygen uptake of the unsupplemented "myasthenic muscle" was very low. The  $Q_{O_2}$  was 1.4, whereas we have found "normal human skeletal muscle" to have a  $Q_{O_2}$  averaging 3.2, a decrease from the "average normal" of 56 per cent. Furthermore, normal human muscle generally shows a linear rate of oxygen uptake for from one and one-half to two hours, while in this case the respiration fell off markedly after one hour. The addition of succinate, fumarate, citrate, glutamate, cytochrome C, prostigmine, or a muscle juice preparation resulted in an increase of oxygen uptake of about 50 per cent over the  $Q_{O_2}$  of the unsupplemented myasthenic muscle, and of 200 per cent over the four-hour  $U_{O_2}$ . Adding succinate and cytochrome, or succinate and prostigmine, gave rates of oxygen uptake similar to what might be expected with normal, unsupplemented human skeletal muscle. The increases here were of the order of 280 per cent over the  $Q_{O_2}$  of the unsupplemented myasthenic muscle, and 540 per cent over the four-hour  $U_{O_2}$ . The combination of cytochrome and prostigmine was no better than either substance alone.

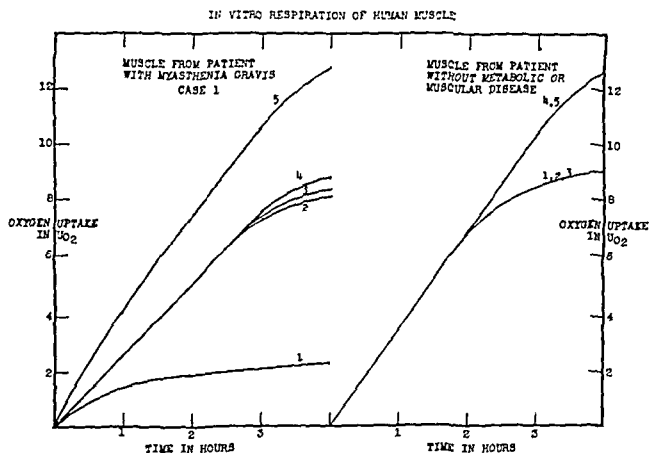


FIG. 1.—The effect of various supplements on the respiration of human muscle. 1, Unsupplemented control; 2, addition of prostigmine; 3, addition of cytochrome C; 4, addition of succinate; 5, addition of succinate and cytochrome.

CASE 2.—A. N., a woman stenographer, aged 26 years, was admitted to Billings Hospital complaining of weakness and stiffness of the fingers and blurring of vision for ten months, fatigue on speaking and chewing for six months, generalized fatigue and inability to open or close her eyes completely for four months, and difficulty in swallowing with regurgitation of fluids through the nose for five weeks. Three weeks before admission she had been told by another physician that she had myasthenia gravis and had been given 6 tablets (presumably 15 mg. each) of prostigmine daily with considerable benefit. She had continued this medication until the day of her entry. Aside from moderate alcoholism the history contained no other relevant material.

General examination, performed after the patient had taken 60 mg. of prostigmine in the preceding eight to ten hours, showed a well-developed and well-nourished woman in no apparent distress. Her height was 163 cm.; weight, 51.7 kilograms; temperature, 100° F. (later normal); and pulse, 92. There were no abnormal findings in the heart, lungs, or abdomen. The blood pressure was 110/70. Five ecchymotic areas were observed over the tibias. Neurologic examination revealed weakness of the orbicularis oculi on both sides, and slight facial weakness. The speech was somewhat mumbling. Ocular convergence

and other extraocular movements were normal. The tone of the skeletal muscles was good. The tendon reflexes were normal. Routine laboratory studies gave essentially normal results except for a slight hypochromic anemia. X-ray of the chest showed no evidence of enlargement of the thymus. The basal metabolic rate, taken forty-one hours after prostigmine and sixteen hours after ephedrine, was -7 per cent.

After admission, prostigmine was withheld for twenty-two hours. At the end of this time there was marked ptosis of the eyelids, which could not be completely closed. Retraction of the lips was limited. The voice became nasal when the patient counted to 70. Definite weakness of the skeletal muscles, especially the deltoids, was observed. Tests with the dynamometer showed R 30, L 27. The patient was then given 0.5 mg. of prostigmine intramuscularly. Within twenty minutes, definite, and in some instances marked, improvement in the above functions took place, and at thirty minutes the dynamometer readings were R 62, L 56.

For the next three days the patient received from 50 to 75 mg. of ephedrine sulfate daily but no prostigmine. The voice became nasal, hand grasp very weak, smile snarling, and fatigue marked. On the fourth day she received neither drug until after the biopsy of the gastrocnemius which was performed late that morning. The surgical procedure and the treatment of the tissue were the same as in Case 1 except for the preoperative administration of 0.18 Gm. of calcium nembutal per rectum. The results are given in Table II. These data differ somewhat from those in Case 1. The oxygen uptake of the unsupplemented myasthenic muscle was higher than in the preceding experiment, but it was still lower by about 18 per cent than values obtained with normal human muscle. The only single supplement which increased the oxygen uptake appreciably was a dicarboxylic acid, fumarate. Neither prostigmine nor ephedrine alone gave a significant change; however, their addition together resulted in a prolongation of the initial rate of oxygen consumption so that the four-hour  $U_{O_2}$  was increased by 22 per cent. Cytochrome C alone was without effect, and its addition with fumarate did not stimulate respiration any more than did the latter substance by itself. The data obtained using a pyruvate substrate were similar to those with glucose, except that here prostigmine alone gave an increase in the four-hour oxygen uptake of 24 per cent.

TABLE II. THE EFFECT OF VARIOUS SUPPLEMENTS ON THE RESPIRATION OF MUSCLE IN MYASTHENIA GRAVIS—CASE 2 (A. N.)

SUPPLEMENTS	OXYGEN UPTAKE (TOTAL $U_{O_2}$ VALUES)			
	1ST HR.	2ND HR.	3RD HR.	4TH HR.
0.01 M glucose in buffer				
O—Control (Values are average of duplicate runs)				
Fumarate	2.8	5.3	7.3	8.6
Fumarate + cytochrome	3.6	8.5	12.6	15.5
Cytochrome	3.7	8.6	12.3	15.5
Prostigmine	2.9	6.2	8.4	9.4
Fumarate + prostigmine	2.7	5.6	8.2	9.3
Ephedrine	3.8	8.8	12.1	15.1
Ephedrine + prostigmine	2.5	5.1	7.1	8.2
	2.8	6.1	8.6	10.6
0.01 M pyruvate in buffer				
O—Control (Values are average of duplicate runs)				
Fumarate	2.4	5.1	6.7	8.0
Cytochrome	4.4	9.4	13.0	15.1
Prostigmine	2.7	5.7	7.6	8.8
Ephedrine	2.7	5.9	8.3	10.9
	2.7	5.4	7.3	9.0

#### DISCUSSION

The results in these experiments differ in some respects from those obtained in similar studies previously reported.<sup>1</sup> In the present cases the  $Q_{O_2}$  of the unsupplemented myasthenic muscle was lower than normal and the addition of dicarboxylic acids produced a definite increase in oxygen consumption, whereas in the earlier experiments the control  $Q_{O_2}$  was normal and it was

not appreciably augmented by the addition of succinate. The difference may have been due to the fact that, in the observations reported in this paper, prostigmine and ephedrine were withheld for at least twenty-four hours before the biopsy, thus rendering the patients definitely "myasthenic," while in the previous experiments prostigmine was not withdrawn and actually was given two hours before the biopsies were performed.

In Case 1 the most interesting feature is certainly the very low respiration of the unsupplemented tissue and the rapidity with which the rate of oxygen consumption fell off after the first hour. In these respects the behavior of the muscle *in vitro* might be considered as exemplifying the clinical condition of the patient at the time of biopsy. Weakness was quite apparent, and an electrocardiogram showed abnormalities which were much less marked in a subsequent tracing taken after treatment with prostigmine and ephedrine had been resumed.

In Case 2, while the oxygen consumption of the unsupplemented tissue was still below normal, it was not as low as in the first case, despite the fact that the second patient was less tolerant of the withdrawal of prostigmine. This, of course, suggests that the decreased oxygen uptake of the respiring muscle is not proportional to the severity of the disease or to the clinical requirement for prostigmine. It may well be asked whether the depression in muscle respiration is reflected in a subnormal consumption of oxygen by the patient as a whole. Unfortunately, no determinations of the basal metabolism were made in Case 1 at this time, though rates several years previously when the patient was having severe symptoms had been within normal limits. Generally speaking, patients with myasthenia gravis do not have a low metabolism, and the basal metabolic rate in the second patient was -7 per cent. We have, then, a situation similar to that involving diabetes and the action of insulin,<sup>2</sup> in which abnormalities in tissue respiration can be demonstrated without corresponding changes in the respiratory metabolism of the intact organism. The explanation of these discrepancies must await further knowledge which may enable us better to correlate the results of *in vitro* studies in general with those obtained from the intact individual.

It is of interest that in Case 1, prostigmine, and in Case 2, prostigmine and ephedrine, both of which were clinically effective in our patients, significantly increased the oxygen consumption of the respiring muscle. In several experiments with normal human muscle such effects have not been observed.

The number of experiments is too small and their scope too narrow to draw any definite conclusions regarding the nature of the lesion in myasthenia gravis. It may be pointed out, however, in connection with the theory postulating a defect in the myoneural junction, that in these *in vitro* studies the observed effects of various supplements were most likely a result of a change in the metabolism of the muscle itself.

#### SUMMARY AND CONCLUSIONS

Experiments have been performed on the respiration of excised skeletal muscle from two patients with myasthenia gravis. In both patients the oxygen consumption of the muscle was lower than normal. It was increased in one by the addition of prostigmine and in the other by prostigmine and ephedrine added together. It was also increased by certain dicarboxylic acids, cytochrome C, and various combinations of these supplements. The results indicate that in this disease the affected muscles show abnormalities in intermediary metabolism.



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## THE EFFECT OF INSULIN ON THE IN VITRO RESPIRATION OF HUMAN SKELETAL MUSCLE

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THE action of insulin on the respiration of muscle in vitro has been studied by a number of workers with results which may be summarized as follows:

*Normal Muscle.*—Insulin has been found to prolong the oxygen uptake of normal pigeon breast muscle mince respiring in various types of phosphate buffers.<sup>1-4</sup> Preparations of insulin rendered inert by heat or alkali are inactive in this respect.<sup>5</sup> As judged by the respiratory quotient, this prolonged oxygen uptake represents normal respiration.<sup>5</sup> This effect of insulin on pigeon breast muscle appears to be connected with the action of certain dicarboxylic acids (fumaric acid, etc.) since it is abolished by malonate which in certain concentrations likewise inhibits the catalytic action of the dicarboxylic acids.<sup>5</sup> In muscle from mice and frogs, Buchner and Grafe<sup>6</sup> found that insulin increased both the oxygen uptake and the respiratory quotient. In other experiments with normal mammalian muscle, however, namely, skeletal muscle of the dog, cat, and rabbit and cardiac muscle of the dog, insulin has been found to have no effect on the respiratory metabolism.<sup>7</sup>

*Diabetic Muscle.*—Stare and Baumann<sup>5</sup> reported that the stimulation by insulin of the oxygen consumption of normal pigeon breast muscle is definitely augmented by pancreatectomy, though Stadie, Zapp, and Lukens<sup>4</sup> did not find this effect. The addition of insulin to preparations of muscle of depancreatized dogs and cats did not result in any increase in the consumption of oxygen or any rise in the respiratory quotient.<sup>7</sup>

The effect of insulin on human muscle, so far as we are aware, has not been studied under these conditions. It is the purpose of this paper to report such experiments.

### EXPERIMENTAL

Muscle of both diabetic and nondiabetic patients was investigated. The diabetic patients were of three types:

#### Group I (*Uncontrolled, insulin-sensitive*)

These patients (Cases 1-6) showed acidosis, ketosis, or marked hyperglycemia. They were all known or proved to be very sensitive to the hypoglycemic effects of insulin. The diabetes in each case was severe.

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*Group II (Controlled, insulin-sensitive)*

The single patient in this group (Case 1, formerly in Group I), was well controlled with insulin and was normally insulin-sensitive.

*Group III (Uncontrolled, insulin-insensitive)*

These patients (Cases 1, 7, and 8) were relatively resistant to insulin. Injections of the hormone were discontinued several days before biopsies were performed. The patient in Case 1 (formerly of Group I, then of Group II) had become resistant for reasons unknown. The other two patients of this group were acromegalic.

A sample of the gastrocnemius muscle weighing about 5 Gm. was removed under local anesthesia with novocain, great care being taken not to infiltrate the muscle itself. The excision was, therefore, accompanied by momentary severe pain. The muscle was freed of all obvious fatty or fibrous tissue, chilled on ice, briefly rinsed with ice water, and minced in a cold micro-laticapic apparatus. The mince was collected in a cold Petri dish lined with several layers of filter paper moistened with cold physiologic saline. Samples of tissue, approximately 200 mg. each, were weighed on cellophane slips on a torsion balance. The cellophane containing the sample was placed in the manometric flask and the tissue was dispersed with a wire. Measurements of oxygen uptake were begun approximately thirty minutes after biopsy. They were carried out at a temperature of 37.5° C. for from one to four hours with and without the addition of insulin and certain other supplements, specifically fumaric and glutamic acids and, in one instance, cocarboxylase.

A glucose Ringer-phosphate buffer<sup>3</sup> free of calcium at pH 7.4 was used. It was made up in twice normal concentration and diluted either with water or with supplementary solutions. The phosphate concentration was M/60. The glucose content was 0.2 per cent. All supplementary solutions were made up fresh for each experiment, neutralized to pH 7.4, and used in final concentration of 0.003 M. The insulin, a commercial preparation, "Netin" U-80, was diluted 1 to 4 with water. The flasks containing insulin received four units. The volume of solution in each flask was 2.8 c.c.

## RESULTS

The data obtained from the six cases in Group I, those patients whose diabetes was severe, uncontrolled, but responsive to insulin treatment, are given in Table I. It is observed that the oxygen consumption of the unsupplemented muscle tissue was below normal in two cases (1 and 2) and in these it was raised to normal by the addition of insulin. The  $Q_{O_2}$  of the muscle from these two cases was 1.9 and 2.4 and in the presence of insulin was 2.8 and 3.1. The dicarboxylic acids also produced an increase in metabolism, and this was further augmented by the addition of insulin. These increases, occurring as they did during the first hour ( $Q_{O_2}$ ), represent a true stimulation of respiration, and in addition the rate of respiration was maintained for a two-hour period. The oxygen consumption of the muscle from the other four cases in this group was not increased by the addition of insulin alone. Generally the addition of a dicarboxylic acid (fumarate or glutamate) prolonged the rate of respiration and in Cases 3 and 4 this effect was observed only in the presence of insulin. It is interesting that in Cases 3 and 4 the presence of insulin alone actually produced some inhibition of oxygen uptake. In the muscle from Case 5 cocarboxylase was added to half of the vessels, and it was found that in the presence of this coenzyme insulin prolonged the rate of respiration whereas it did not do so in the absence of cocarboxylase. The experiment

TABLE I. PROTOCOLS\* ON THE RESPIRATION OF MUSCLE FROM DIABETIC PATIENTS IN GROUP I  
(UNCONTROLLED, INSULIN-SENSITIVE)

Case 1 (Lap.), aged 54 years; severe diabetes; duration, fourteen years; marked weakness; never received insulin; blood sugar, 263 mg. per 100 c.c.; no ketosis

SUPPLEMENTS	OXYGEN UPTAKE IN $UO_2$		
	1 HR.	1½ HR.	2 HR.
None (control)	1.9	3.0	3.9
Insulin	2.8	4.5	6.0
Fumarate	2.9	4.7	6.3
Insulin + fumarate	3.4	5.6	

*Comment.*—The control  $Q_{O_2}$  is low. Respiration was increased by insulin, also by fumarate, but fumarate + insulin were better than either alone.

Case 2 (Wi.), aged 16 years; severe diabetes; duration, eight months; poorly controlled with inadequate insulin; blood sugar at biopsy 550 mg. per 100 c.c.; acidosis

SUPPLEMENTS	OXYGEN UPTAKE IN $Q_{O_2}$	
	1 HR.	
None (control)	2.4	
Insulin	3.1	
Fumarate	3.1	
Insulin + fumarate	3.1	
Glutamate	3.0	
Insulin + glutamate	3.8	

*Comment.*—Insulin, fumarate, and glutamate increased respiration about equally. Insulin + fumarate were no better than either alone, but insulin + glutamate were more effective than glutamate alone.

\*All results are meters of oxygen consumed during the consumed at the end of the period designated.

Case 3 (Al.), aged 25 years; severe diabetes; duration, ten years; fair control; insulin withdrawn 5:00 P.M., December 5; on December 7 marked acidosis necessitated 80 units of insulin between 1:00 A.M. and 6:00 A.M.; biopsy 1:00 P.M., December 7, with blood sugar of 86 mg. per 100 c.c., but patient still nauseated

SUPPLEMENTS	OXYGEN UPTAKE IN $UO_2$		
	1 HR.	2 HR.	3 HR.
None (control)	3.6	6.4	7.8
Insulin	3.4	5.2	5.8
Insulin added at one hour	3.4	5.3	6.0
Fumarate	4.3	6.1	6.7
Fumarate + insulin at one hour	4.0	8.6	11.6

*Comment.*—Insulin alone produced some depression of respiration, but when added with fumarate caused a considerable increase at two and three hours.

Case 4 (St.), aged 47 years; severe diabetes; duration, eight years; fairly good control; insulin withdrawn 8:00 A.M., March 30; marked acidosis necessitated fluids and 50 units of insulin at 11:00 P.M., March 31, and 15 units at 3:00 A.M., April 1; biopsy at 8:50 A.M. on this day with blood sugar 431 mg. per 100 c.c. and urine showing large amounts of ketone bodies

SUPPLEMENTS	OXYGEN UPTAKE IN $UO_2$		
	1 HR.	2 HR.	3 HR.
None (control)	3.0	5.5	6.9
Insulin	2.6	3.6	4.0
Fumarate	3.2	6.4	7.3
Fumarate + insulin	3.4	7.2	9.0

*Comment.*—Insulin alone produced definite inhibition of respiration but when added with fumarate augmented the slight increase produced by the latter.

TABLE I—CONT'D

Case 5 (Eg.), aged 20 years; severe diabetes; duration, two years; poor control; insulin withdrawn March 20; biopsy March 21, with blood sugar 345 mg. per 100 c.c. and patient in ketosis; clinical and chemical acidosis four hours later

SUPPLEMENTS	OXYGEN UPTAKE IN $UO_2$			
	1 HR.	2 HR.	3 HR.	4 HR.
None (control)	3.1	5.7	6.5	6.8
Coccarboxylase	2.9	5.6	7.0	7.9
Insulin	3.0	5.0	5.6	6.0
Insulin + coccarboxylase	3.0	5.8	8.6	9.5
Insulin at two hours	3.0	6.0	7.0	7.4
Coccarboxylase + insulin at two hours	3.0	5.8	8.7	9.5
Fumarate	3.5	7.4	11.2	14.1
Fumarate + coccarboxylase	3.3	7.1	10.7	13.0
Insulin + fumarate	3.1	6.3	9.5	11.6
Insulin + fumarate + coccarboxylase	3.5	6.7	10.0	13.2

*Comment.*—Insulin had no effect on the respiration when only the customary glucose-Ringer-phosphate buffer was used. When coccarboxylase was added, a moderate increase was produced by insulin. This was manifested chiefly as a prolongation of the original respiratory rate. Thus, if only the oxygen consumed during the last two hours of each experiment is considered, it is found that the control tissue used  $7.0 - 5.6 = 2.3$  c.c. of oxygen per milligram of dry weight of tissue, whereas the tissue to which insulin was added consumed  $9.5 - 5.8 = 3.7$  c.c., an increase of 62 per cent. Fumarate was ineffective in facilitating the action of insulin.

Case 6 (Am.), aged 21 years; moderately severe diabetes; duration, three months; never received insulin; blood sugar at biopsy, 338 mg. per 100 c.c., and patient in ketosis but not acidosis

SUPPLEMENTS	OXYGEN UPTAKE IN $UO_2$			
	1 HR.	2 HR.	3 HR.	4 HR.
None (control)	3.4	6.7	8.5	9.1
Insulin	3.5	6.3	7.5	8.0
Fumarate	3.9	8.3	12.6	16.2
Fumarate + insulin	3.9	8.0	11.7	15.0
Glutamate	4.2	8.8	13.0	16.4
Glutamate + insulin	4.2	8.4	12.0	14.5

*Comment.*—No effect was produced by insulin, either alone or with other supplements.

with muscle from Case 6 is of interest because the respiration was unusually good. The  $UO_2$  even at three or four hours and the response of the muscle to fumarate and glutamate compared favorably with what one would expect with "normal" human skeletal muscle. Insulin was without effect either alone or when added to a dicarboxylic acid.

Only one experiment was done with muscle from a patient representative of Group II, that is, a controlled diabetic patient responsive to insulin therapy. The data from this experiment are given in Table II. It is observed that the unsupplemented muscle had a  $QO_2$  of 2.6 which is on the low side of what we have found for supposedly normal human skeletal muscle. Insulin was without effect either alone or in the presence of fumarate.

In Table III are given the results with muscle obtained from patients in Group III who were uncontrolled diabetic patients and not responsive to insulin. In these three experiments insulin produced no significant effect except in muscle from Case 8 where it definitely inhibited oxygen consumption. It is also interesting that the muscle from this last case had a very low  $QO_2$  and that it did not respond in the usual manner to dicarboxylic acid supplements.

The data secured in one subject (Case 1), who passed successively through all the phases of diabetes represented by Groups I, II and III, and in whom muscle biopsies were obtained during each stage, are of particular interest.

TABLE II. PROTOCOLS\* ON THE RESPIRATION OF MUSCLE FROM DIABETIC PATIENTS IN GROUP II (CONTROLLED, INSULIN-SENSITIVE)

Case 1 (same as Case 1, Group I); well controlled for three weeks with from 50 to 90 units insulin per day; allergic reactions at injection sites avoided by intramuscular route; insulin not withdrawn prior to biopsy

SUPPLEMENTS	OXYGEN UPTAKE IN $UO_2$	
	1 HR.	1½ HR.
None (control)	2.6	4.4
Insulin	2.7	4.5
Fumarate	3.2	5.2
Insulin + fumarate	3.4	5.7

*Comment.*—No appreciable effect was produced by insulin.

\*All results are the average of duplicate determinations and are given as cubic millimeters of oxygen consumed per milligram of dry weight of tissue. The expression  $UO_2$  indicates the total amount of oxygen consumed at the end of the period designated.

TABLE III. PROTOCOLS\* ON THE RESPIRATION OF MUSCLE FROM DIABETIC PATIENTS IN GROUP III (UNCONTROLLED, INSULIN-INSENSITIVE)

Case 1 (same as Case 1, Groups I and II); definitely resistant to insulin for three months, requiring from 400 to 1,200 units (average about 725 units) per day; insulin withdrawn for fifty-one hours except for 15 units (acidosis) given fifteen hours before biopsy; blood sugar, 390 mg. per 100 c.c.; moderate ketosis

SUPPLEMENTS	OXYGEN UPTAKE IN $UO_2$		
	1 HR.	2 HR.	3 HR.
None (control)	2.7	4.3	4.7
Insulin	2.8	4.0	4.4
Fumarate	4.0	6.1	6.7
Insulin + fumarate	3.5	5.1	5.5

*Comment.*—No significant effect was produced by insulin.

Case 7 (Cr.), aged 33 years; acromegaly, four years; diabetes, two years, requiring from 150 to 300 units insulin per day; insulin withdrawn seventeen days before biopsy; blood sugar, 266 mg. per 100 c.c.; glycosuria, 4 plus; no ketosis

SUPPLEMENTS	OXYGEN UPTAKE IN $UO_2$			
	1 HR.	2 HR.	3 HR.	4 HR.
None (control)	3.0	5.8	7.6	8.2
Insulin	3.2	6.0	7.4	7.7
Fumarate	3.5	7.1	9.9	11.4
Fumarate + insulin	3.7	7.9	11.0	12.5

*Comment.*—Insulin neither alone nor with fumarate produced any significant change in the respiration.

Case 8 (Lan.), aged 57 years; severe diabetes; duration, twelve years; control fair to good; acromegaly six years; also pyelonephritis and neuritis; for three days before biopsy, insulin dose, previously 55 units per day, reduced to 20 units per day; blood sugar at time of biopsy, 419 mg. per 100 c.c.; no ketosis

SUPPLEMENTS	OXYGEN UPTAKE IN $UO_2$	
	1 HR.	2 HR.
None (control)	1.5	4.0
Insulin	1.1	2.7
Fumarate (0.007 M.)	1.9	3.2
Fumarate + insulin	1.4	3.8
Glutamate (0.007 M.)	1.6	4.1
Glutamate + insulin	1.7	4.2

*Comment.*—The  $QO_2$  is very low. Insulin alone definitely depressed respiration. Insulin with other supplements had no significant effect.

\*All results are the average of duplicate determinations and are given as cubic millimeters of oxygen consumed per milligram of dry weight of tissue. By  $QO_2$  is meant the oxygen consumed during the first hour. The expression  $UO_2$  indicates the total amount of oxygen consumed at the end of the period designated.

At a time when this patient had not received treatment of any kind and when the blood sugar was high, the oxygen consumption of the unsupplemented muscle was low and was brought up to normal by the addition of insulin to the Warburg flask (Table I). After the disease had been adequately treated, the in vitro respiration of the unsupplemented tissue was higher and well sustained but was not increased by the addition of insulin to the medium (Table II). Similar results were obtained in a third experiment several months later when the patient, now highly resistant to insulin and requiring from 400 to 1,200 units per day, had lapsed from control.

It is difficult in experiments of this type to have proper controls and to be able to compare the results with similar studies done on supposedly normal skeletal muscle. For examples of the latter, we have usually used biopsies of the abdominal rectus, gluteus maximus, and gastrocnemius obtained at routine surgery from individuals without known metabolic or muscular disease. As a result of some forty experiments of this type, we have found that these samples of skeletal muscle are alike in their in vitro oxygen uptake and response to various supplements. The  $Q_{O_2}$  averages between 2.5 and 4.0, and a linear rate of oxygen uptake is maintained for from one and one-half to two hours. Response to dicarboxylic acids is similar to that obtained from skeletal muscle from other species,<sup>3</sup> and insulin is without effect on the in vitro oxygen uptake.

#### SUMMARY

Insulin has been found to increase and prolong the rate of in vitro oxygen consumption of minced skeletal muscle excised from severely diabetic patients whose diabetes was uncontrolled but responsive to insulin therapy. In two patients the effect was observed without the addition of other supplements; in two patients it was observed only after a dicarboxylic acid had been added to the medium; in one patient it was necessary to have added coenzyme; and in one patient no insulin effect either alone or in the presence of a dicarboxylic acid was found.

Insulin was without effect on the in vitro respiration of skeletal muscle from a controlled insulin-sensitive diabetic patient or from three patients whose diabetes was uncontrolled and not readily responsive to insulin therapy. Likewise, insulin gave no effect on the in vitro respiration of skeletal muscle from patients without metabolic or muscular disease.

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## THE EFFECT OF ULTRAVIOLET IRRADIATION ON THE BLOOD HEMOGLOBIN

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THE influence of ultraviolet radiation on the formation of hemoglobin and erythrocytes and its effectiveness in the treatment of anemia have not been clearly established. The literature reveals much conflicting evidence with few controlled clinical experiments bearing on the subject. It has been shown that repeated irradiation to animals stimulates hematopoiesis,<sup>1, 2</sup> causing an unusually rapid production of erythrocytes. Mayerson and Laurens have shown that irradiation with the flaming carbon arc and the mercury quartz lamp produces a marked increase in the erythrocytes and reticuloocytes in dogs made anemic by hemorrhage<sup>3</sup> as well as in dogs in which a hemolytic anemia was produced by the administration of acetylphenylhydrazine (pyrodine).<sup>4</sup> Ultraviolet irradiation has also been reported to be effective in raising the hemoglobin level in rats rendered anemic by a milk diet.<sup>5</sup>

There have been many reports concerning the effect of ultraviolet irradiation on the anemia of human subjects. Kovacs<sup>6</sup> observed its effect on fifty-nine patients whose anemia was secondary to a wide variety of diseases. He noted a slight increase in the blood hemoglobin in fifteen of the thirty-seven patients treated and an increase in the erythrocytes in seventeen patients. The effects were not striking. In a subsequent report<sup>7</sup> he concluded that the production of hemoglobin is accelerated and the number of erythrocytes is increased by ultraviolet irradiation. It has been shown that this form of therapy causes an increase in the blood hemoglobin in debilitated children,<sup>8</sup> but Laurens<sup>9</sup> and Krusen<sup>10, 11</sup> believe that although the radiation may have some effect on certain forms of anemia, its usefulness is limited, its effects are nonspecific, and it serves only as a useful adjunct to other forms of treatment. Ultraviolet irradiation is of proved value in the treatment of certain conditions such as rickets, bone and joint tuberculosis, and some other disease which may be accompanied by an anemia. As the primary disease is improved by this treatment, there is a concomitant improvement in the anemia.

In an attempt to evaluate the effect of ultraviolet irradiation on hemoglobin formation, we selected forty subjects with mild, but uncomplicated, anemia. These patients were students and hospital personnel who were in good health and whose history and physical examination revealed no recognizable disease except for the low blood hemoglobin. We have encountered many similar subjects whose hemoglobin level was below the commonly accepted standards of normal but in whom there was no recognizable cause for the anemia. It has been our belief<sup>12</sup> that this low level of hemoglobin represents a normal level for some individuals, whereas in others a similar hemoglobin level represents a mild grade of anemia which, in the absence of other causative factors, may be on a nutritional basis. The administration of iron salts to such subjects produces an increase in their hemoglobin level which reaches a peak at the end of from eight

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to ten weeks and then falls. The blood hemoglobin may drop to the pre-treatment level in the nonanemic subjects or to a point slightly above this level in those with mild anemia. This type of subject was selected for irradiation therapy in the belief that any stimulating effect which the rays might have would become evident in as much as there were no complicating diseases or deficiencies in the individuals and the bone marrow was apparently normal. Iron salts had previously been administered to many of the women and to some of the men subjects without significant or persistent benefit. All the subjects ate an adequate and well-balanced diet, but by reason of their occupation they were indoors much of the time without exposure to sunshine.

These observations were made during the fall and winter months of the year when there is a tendency, in this locality, for a slight but gradual decrease in the blood hemoglobin. As a control, a group of thirty-two subjects (twenty-five women and seven men) were selected whose hemoglobin levels corresponded to those of the group under treatment. The control subjects were observed during the same months as the group under treatment, their environment and eating habits were essentially the same, and they received no medication or treatments.

The radiation used in this study was obtained from a Burdick air-cooled quartz lamp. The burner of the lamp was placed thirty inches above the body

TABLE I

SUBJECT	INITIAL BLOOD	WEEKLY BLOOD HEMOGLOBIN (GM.)									
	HEMOGLOBIN GRAMS	I	II	III	IV	V	VI	VII	VIII	IX	X
Men											
J. D.	12.77	+1.86	+1.36	+ .87							
R. P.	12.34	- .19	+ .81	- .06	+1.05	+1.05					
E. P.	12.34	-	-1.05	+ .19	- .31	-	+ .56				
E. A.	12.77	+ .12	+1.12	+ .12	± .00	+ .74	- .56				
R. B.	13.89	+ .12	+ .50	+ .50	+ .99	-	- .74	+ .12			
P. B.	13.39	- .25	- .12	+ .25	+ .37	-	+ .37	-	+1.98		
D. M.	13.89	+ .50	+1.12	+ .75	+ .99	-	-	+ .25	+1.49	+1.98	
L. S.	12.77	- .37	- .12	-	+ .87	- .12	+ .75	+ .25	-	-	+ .62
R. A.	13.64	+1.36	- .50	+ .50	-	+ .99	+ .62	+ .62	± .00	- .99	- .83
G. H.	12.90	+ .12	+1.61	+1.24	+ .50	+1.12	- .62	- .87	- .76	+ .99	-
S. S.	12.90	+1.86	+ .62	+ .74	+1.49	-	+2.73	+2.23	+ .87	+ .99	+ .62
J. N.	12.09	+1.80	-	+ .68	+ .68	+ .93	-	-	-	-	+1.55
O. L.	12.77	-	+1.24	± .00	-	+ .87	+1.61	- .25	+ .50	+ .50	+1.24
Women											
J. W.	9.77	-	+ .30								
V. T.	9.17	+ .90	-	+1.77							
H. B.	12.21	+ .81	+ .56	- .25	- .81						
J. D.	12.40	- .74	- .50	-	+ .12						
P. C.	9.36	-	-	-	-	-	+ .75				
R. K.	11.28	-	- .31	-	- .31	-	- .62	+ .19			
P. L.	9.80	-	+ .68	-	+ .68	+1.24	+ .56	+ .31	+ .43		
M. P.	9.92	-	-	+ .50	-	+ .43	-	-	-	+ .81	
A. D.	10.04	+ .93	+ .88	-	-	-	-	-	+ .42	+1.12	
M. C.	10.23	+ .12	+1.12	+ .12	+ .81	+ .31	+ .93	+ .31	+ .43	+1.12	
L. G.	10.35	± .00	+1.24	-	-	+ .06	+ .50	-	- .43	- .12	
B. H.	11.72	+ .62	+ .68	+ .46	+ .93	+ .31	+ .93	+1.18	-	+1.05	
W. B.	10.66	+ .62	-	+ .25	-	+2.73	+1.36	+ .93	-	-	+ .50
G. G.	9.24	-	-	+ .68	+ .93	-	-	+ .12	- .68	-	-
E. H.	10.66	+ .37	-	+1.36	-	+ .99	-	-	-	-	+ .99
R. K.	12.77	+ .37	+ .37	- .56	-1.43	+1.24	-	-	-1.18	-1.24	-
F. C.	11.04	- .56	+ .93	-	+ .74	± .00	- .06	+ .12	+1.36	+1.05	-
E. Z.	11.47	+1.18	-	+ .56	+ .81	+1.55	-	-	+1.20	+1.05	+2.29
M. W.	11.72	-	+1.55	+ .56	- .37	+ .50	+1.30	+ .93	+ .06	+ .62	+ .81
E. Y.	12.40	± .00	-	- .62	-	± .00	-	- .12	+ .87	-	-
L. C.	12.52	-	± .00	+ .87	- .12	+ .75	+ .25	+ .62	- .66	+ .19	- .43
L. M.	12.65	- .93	- .56	-1.55	- .62	- .43	- .50	+ .62	+ .74	+1.05	-1.47
E. C.	12.09	- .56	- .87	- .93	-1.67	- .81	- .87	-	± .00	-	-
E. J.	10.85	+ .93	-	-	+ .06	-	- .31	-	- .31	± .00	- .31
E. H.	10.48	+ .31	+ .43	+ .62	+ .99	+ .50	+ .87	-	+ .68	-	-
M. P.	10.54	+ .31	-	-1.12	-	- .12	-	-	- .87	-	+ .19
E. H.	9.02	-	+ .31	-	+ .90	-	+1.20	-	-	+1.35	-



and the shutters were adjusted so as to concentrate the light on the exposed portion of the body. The erythema dose was determined for each individual, being about thirty seconds in most cases, and this amount of irradiation was given as the initial exposure on both the anterior and posterior surfaces of the body, respectively. Barring reactions, the amount of irradiation was increased thirty seconds with each of the three treatments per week until a maximum exposure of seven minutes on both the ventral and dorsal surfaces was reached. This exposure was given for one week, then reduced to thirty seconds, and again built up in the same manner. In the few instances in which a significant erythema developed, the exposure time was decreased thirty seconds, held at that level for several treatments, and then increased in the usual manner.

Blood hemoglobin determinations were made at weekly intervals, using an acid hematin method with a Klett-Summerson photoelectric colorimeter.

The results of the weekly blood hemoglobin determinations are shown in Table I and the change in the hemoglobin levels for the treated and the control groups are illustrated graphically in Fig. 1.

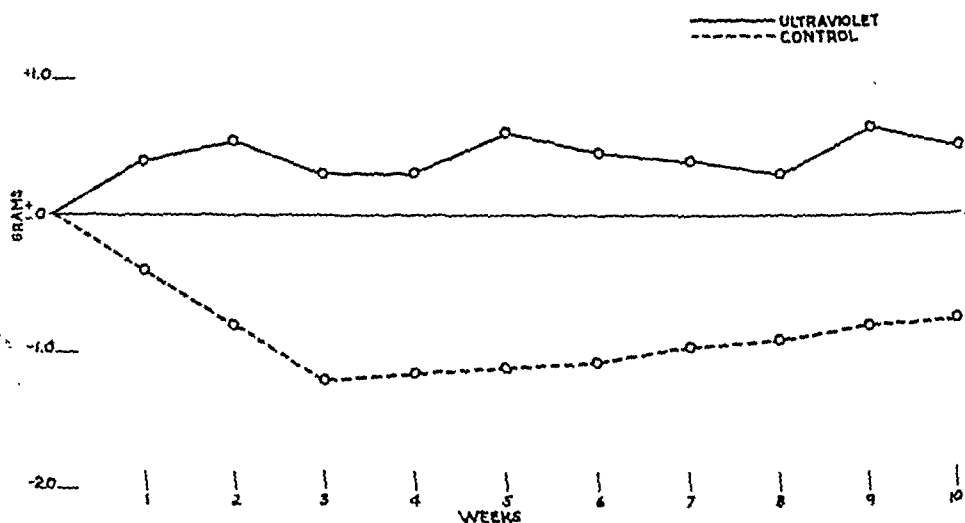


Fig. 1.—Effect of ultraviolet irradiation on blood hemoglobin level.

It will be noted in Table I that a majority of the subjects, both men and women, who received ultraviolet irradiation showed a rise in blood hemoglobin. This was not a striking increase, but only a moderate elevation in all cases. The increase in hemoglobin is portrayed graphically in Fig. 1 as the average increase for the group. It will be noted that although there is a slight increase in the hemoglobin level which is maintained throughout the observation period, there is not a progressive and continuing rise. Hemoglobin readings made at weekly intervals on any group of individuals will show variations which are as great as the variations encountered in this group of subjects, but they will not show the persistent increase. The results assume greater significance when compared to the control group and when it is realized that the observations were made during a time of year when there is a normal tendency in this locality for a slight reduction in blood hemoglobin.

#### CONCLUSION

These results indicate that ultraviolet irradiation produces a very slight but persistent increase in the blood hemoglobin level. The increase is not great

enough to recommend this as a routine therapeutic procedure in hypochromic anemia, although it may sometimes be useful as a supplement to other forms of therapy in selected cases.

The Burdick Corporation of Milton, Wis., kindly furnished the Quartz Mercury Ultraviolet lamp used in this study, a Burdick Professional Special.

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## THE EFFECT OF DIGITALIS, EPINEPHRINE, AND SURGERY ON THE RESPONSE TO HEPARIN

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THE current interest in anticoagulants in the prevention of thrombosis has led to renewed discussion of the effects of various agents on the coagulation time.<sup>1-5</sup> Recently de Takats<sup>6</sup> has described a heparin tolerance test for use in the study of the coagulation mechanism and has commented on the effect of various agents on the response to heparin.<sup>1, 4-6</sup> This study was carried out to determine in our own experience the effects of epinephrine, digitalis, and major surgery on the heparin tolerance test.

#### METHODS

In measuring heparin tolerance it is of prime importance that the coagulation time be followed by a method capable of accurately revealing small changes. The methods of Howell and of Lee-White, while suitable for many clinical purposes, do not detect relatively small changes in the coagulation

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The patients used in this study were obtained through the courtesy of Dr. A. H. Colwell and the attending staff of The Presbyterian and Woman's Hospitals of the University of Pittsburgh.

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time<sup>7</sup> and therefore were not used in this study. Kruse's method, which indicates the initiation of coagulation as contrasted to the relatively complete coagulation obtained with the Lee-White procedure, was found in preliminary work to be a satisfactorily sensitive test of the coagulation time.<sup>7</sup>

After control coagulation times had been established, a dose of sodium heparin of 0.15 mg. per kilogram body weight was administered intravenously. The coagulation time was then determined at 2- to 5-minute intervals until it had returned to control levels. A fresh skin puncture was used for each determination.

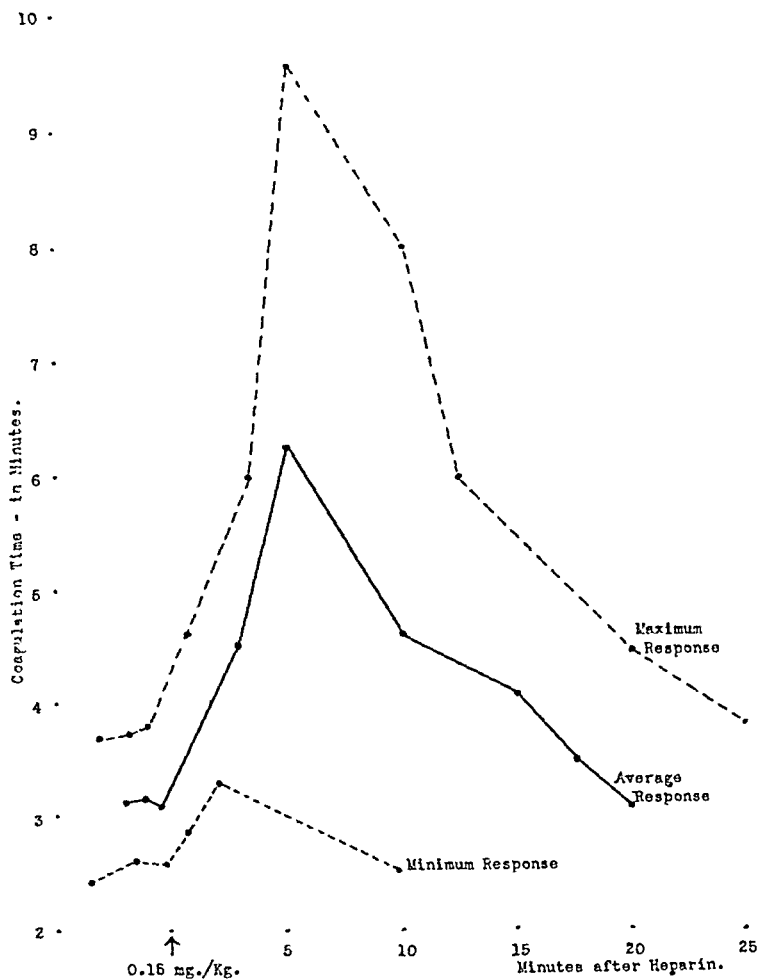


Fig. 1.—Average, maximum, and minimum response to 0.15 mg. heparin per kilogram of body weight.

#### RESULTS

*Control Experiments.*—Control coagulation times in the twenty-five individuals tested varied from 2.5 to 5 minutes. Following the administration of 0.15 mg. of heparin per kilogram of body weight the coagulation time rose from 1 to 8 minutes above the control level within 10 minutes after injection. In all control individuals the coagulation time had returned to the base level within 25 minutes. In Fig. 1 is indicated an average response to this dose of heparin as well as the maximum and minimum increase noted in this series. De Takats<sup>8</sup> has previously commented on hypo- and hyperreactors to heparin and de-

scribed a biphasic reaction<sup>1</sup> with a primary and secondary peak in coagulation time. Such biphasic reactions were not observed in this series. Repetition of the tests even after several weeks revealed relatively constant responses. This is demonstrated in Fig. 2.

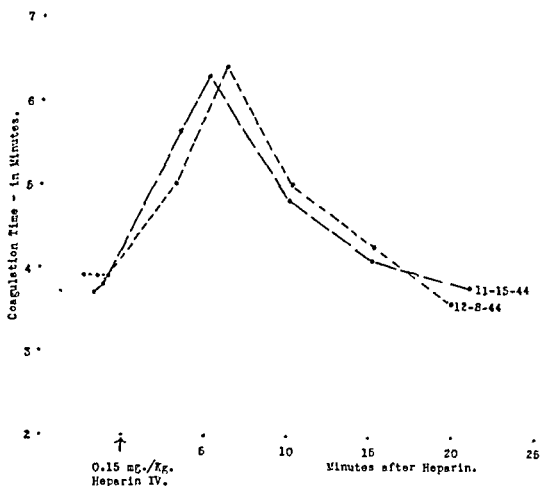


Fig. 2.—Consistent response to administration of 0.15 mg. sodium heparin per kilogram of body weight repeated after three-week interval

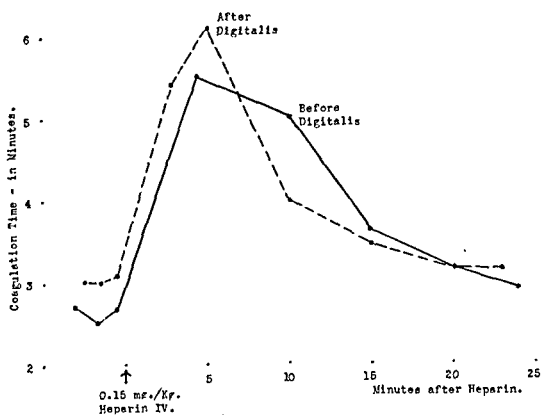


Fig. 3.—Response to heparin before and after the administration of 141 gr. of digitalis in thirty-three days.

*Digitalis Administration.*—Several investigators<sup>2-5</sup> have found a reduction in the coagulation time following the administration of digitalis. This has been reported to be especially evident with the giving of relatively large doses to animals.<sup>2-4</sup> With the use of therapeutic amounts in man<sup>5</sup> the decrease in

coagulation time as compared to the controls was much less evident. The heparin tolerance of eight individuals before, during, and after therapeutic digitalization failed to reveal any significant or consistent decrease in either the coagulation time or the response to heparin in this study. In Fig. 3 is illustrated the response to heparin before and after the administration of 141 gr. of digitalis in thirty-three days.

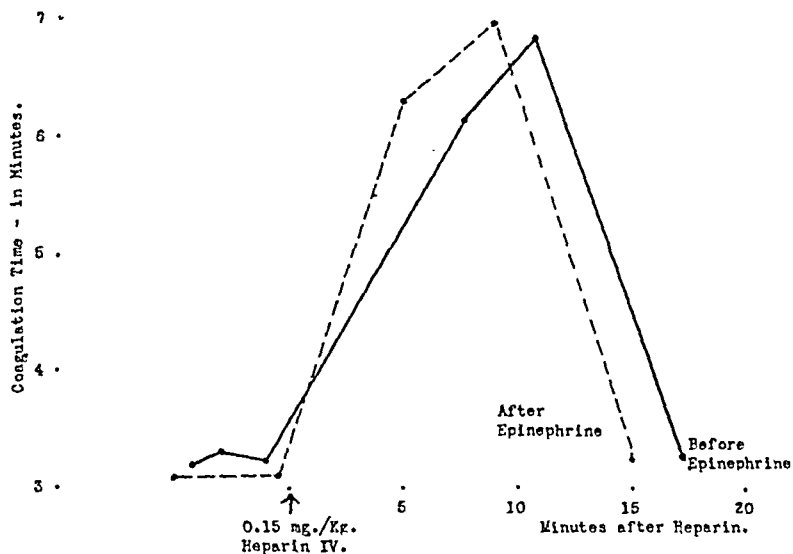


Fig. 4.—Heparin tolerance curve before and after the administration of 1 mg. of epinephrine intravenously.

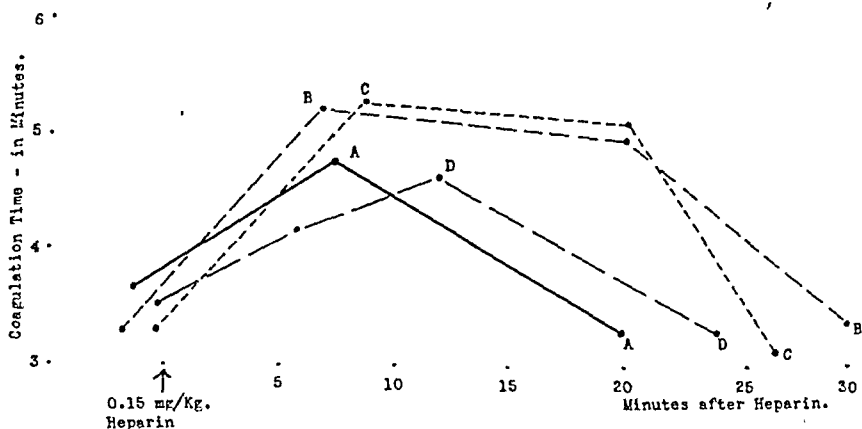


Fig. 5.—Effect of heparin on coagulation time before, A, and three days, B, five days, C, and seven days, D, after subtotal gastrectomy.

**Epinephrine.**—Epinephrine reportedly hastens coagulation of blood but such reports have not been generally confirmed.<sup>8</sup> Recently a decrease in the heparin tolerance curve has been reported following the administration of epinephrine.<sup>1</sup> In five individuals no significant or consistent change in the heparin tolerance curve following the administration of 1 mg. of epinephrine intravenously has been obtained in this study. In Fig. 4 is illustrated a typical response.

**Surgery.**—It is generally recognized that thrombosis is prone to occur in the postoperative period. In nine patients in whom the coagulation time and heparin tolerance was followed before and at intervals after a major surgical

procedure, no significant or consistent decrease has been obtained. In Fig. 5 are indicated the results of the heparin tolerance test before and at three, five, and seven days after subtotal gastrectomy. In this individual an actual prolongation of the heparin tolerance curve was obtained rather than a decrease as observed by others.<sup>6</sup>

#### SUMMARY

A study of the response of individuals to the intravenous administration of 0.15 mg. of heparin per kilogram of body weight is presented.

No significant decrease in heparin tolerance was obtained after the administration of digitalis or epinephrine or after major surgery.

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## LABORATORY METHODS

### A DEMAND APPARATUS FOR AUTOMATIC DELIVERY OF AEROSOLS DURING INSPIRATION

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THE nebulization of bronchodilator and chemotherapeutic aerosols by a continuous stream of oxygen passing through a nebulizer delivers these drugs during both cycles of respiration, thus permitting a loss of the solution during the expiratory cycle.<sup>1, 2</sup> In the clinical use of penicillin aerosol a Y tube was inserted between the regulator and the nebulizer. The open end of the Y tube was closed by application of a finger just before the beginning of inspiration, which forced the oxygen flow of from 4 to 8 liters per minute through the nebulizer, and, before the termination of inspiration, the finger was removed, which permitted the oxygen stream to pass into the outside atmosphere and terminate the nebulization of the penicillin. The aerosol was thereby produced and inhaled during inspiration only. The volume of the nebulizer was enlarged to contain a 1,000 c.c. reservoir bulb to collect penicillin in the exhaled air.<sup>3, 4</sup>

In the apparatus here described the lowering of pressure within the nebulizer at the start of inspiration releases a flow of oxygen from the demand valve which then passes through the nebulizer. The penicillin aerosol is thus formed only during inspiration without the cooperation of the patient being required.

#### DESCRIPTION

The automatic nebulizing demand valve (Fig. 1) consists of an aluminum casing divided into three separate chambers (1, 2, and 3). The first chamber, 1, houses the valve, *B*, which controls the flow of gas to the nebulizer. The second chamber, 2, is kept at atmospheric pressure by means of two holes communicating with the outside air. It is separated from the third, or activating chamber, 3, by a thin rubber diaphragm, *E*, which is stiffened with two thin sheets of plastic, *D*. A valve stem, *C*, connected to the rubber diaphragm, and passing through a bearing into the top chamber, 1, is capped with a head containing a neoprene washer. The valve stem is raised and lowered by movement of the rubber diaphragm and when fully elevated its head makes contact with the valve seat, *B*, interrupting the flow of gas which flows through the outlet, *J*. An ordinary hospital regulator attached to the demand valve by means of the adapter nut, *A*, supplies the air or oxygen flow for the system.

The third, or activating chamber, 3, contains a lever arm, *F*, which is pressed upward against the underside of the diaphragm by means of a spring, *G*. The spring, housed in a gastight cap, may be shortened or lengthened to exert varying degrees of tension against the lever arm. The change in spring tension

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is accomplished by turning the thumbserew, *H*. The outlet, *K*, from the activating chamber permits the transmission of pressure fluctuations to the underside of the diaphragm.

The nebulizer\* is adapted from a type used for administration of 1 per cent epinephrin. A 500 or 1,000 c.c. flask, *L*, is fused to the top of the reservoir, *M*. As air or oxygen flows through the inlet, *P*, it converts the inhalant solution to a very fine suspension of droplets which leave the system through the mouthpiece, *O*. The velocity of gas flow through the reservoir sets up a slight negative pressure and more air is drawn from the outside atmosphere through the restricted orifice, *R*. This latter fraction of air then mixes with the suspension of droplets in *L* and *M* and leaves the nebulizer at the mouthpiece. With this arrangement any droplets of the inhalant remaining in the flask after expiration are drawn into the lung, with the newly formed aerosol, during the succeeding inspiration.

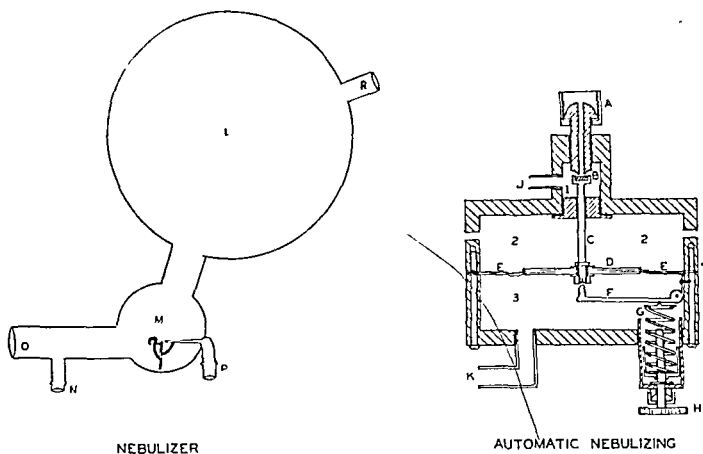


Fig. 1

Outlet *K* of the demand valve is attached to the nebulizer, *N*, by means of a three-foot length of  $\frac{3}{8}$  inch I.D. tubing. The flow outlet *J* is connected to the inlet *P* of the nebulizer with a three-foot length of  $\frac{1}{4}$  inch I.D. pressure tubing.

#### DIRECTION FOR USE

Connect the demand regulator to a hospital flow regulator by means of the adapter nut *A*. Decrease the tension of the spring by turning the thumbserew clockwise until it stops. Turn handle of the hospital regulator to deliver the flow of air or oxygen desired. This flow may be from 4 to 10 liters per minute, depending on the speed of aerosolization or density of aerosol desired. Increase the spring tension against the lower arm by turning the thumbserew counter-clockwise, until the pressure of the lever arm against the diaphragm is just

\*Vaponefrin Co., Upper Darby, Pa.



sufficient to hold the valve stem against the valve seat with sufficient force to cut off the flow of gas from the regulator.

Connect the demand regulator outlets, *J* and *K*, to the nebulizer outlets, *P* and *N*, with the proper tubing. Place 1 or 2 c.c. of the inhalant solution at the bottom of the nebulizer reservoir. Direct the mouthpiece back into the mouth and have the subject hold it firmly with the lips and breathe only through the mouth. As inspiration begins there is a slight fall (1 cm.  $H_2O$ ) in pressure in the flask due to the restricted intake orifice, *R*. This pressure fall is transmitted through the pressure outlets *N* and *K* and is applied to the underside of the rubber diaphragm. Since the upper side of the diaphragm is maintained at atmospheric pressure, the diaphragm will be lowered. In so doing it moves the valve stem downward and permits gas from the hospital regulator to pass through *B* and then chamber *I* and then proceed to the nebulizer inlet, *P*. Aerosolization then takes place and a mixture of the aerosol, and air entering the system through *R*, is drawn into the lungs. At the end of inspiration the pressure in the nebulizer returns to atmospheric, and this pressure transmitted to the activating chamber permits the diaphragm to rise. The rising diaphragm forces the valve stem against the seat and interrupts the flow of gas through the demand valve. During expiration the expired gas passes through the nebulizer reservoir and flask and leaves the outlet *R*. Some of the finely suspended droplets of aerosol are precipitated on the walls of the flask, some leave through *R*, and the remainder stay in suspension in the flask. The latter are then drawn into the lungs, during the succeeding inspiration.

#### SUMMARY

A demand regulator for nebulization of chemotherapeutic aerosols is described which automatically operates during inspiration only. This apparatus has been used for clinical administration of penicillin.

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# DETERMINATION OF HEMOGLOBIN

## THE USE OF A BUFFER AS THE LAKING FLUID TO OVERCOME ERRORS INHERENT IN THE CARBONATE METHOD

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THE rapid yet accurate determination of hemoglobin has for many years been a major clinical laboratory problem. With the advent of photoelectric colorimeters several of the original difficulties were overcome. In this paper, data will be presented which point out a possible error in one of the photometric methods now in general use.

The use of dilute carbonate (0.1 per cent) as the laking fluid for red cells and solvent for hemoglobin dates back to the work of Hüfner<sup>1</sup> and others in the latter half of 1800. In 1929 Sheard and Sanford<sup>2</sup> developed a relatively simple and inexpensive filter photometer primarily for the determination of hemoglobin and recommended the dilution of 0.1 c.c. of blood in 20 c.c. of 0.1 per cent sodium carbonate. Sheard and Sanford, and later workers who have used this method on other instruments, have emphasized the importance of laking and of shaking to insure oxygenation of the hemoglobin but have not discussed a critical time for the reading of the sample. In the photometric method, the light absorptive power of oxyhemoglobin at one of the maxima of absorption (542 mμ) is used as a measure of the amount of pigment present.

It is generally implied that the photometric measurements be made as soon as laking and oxygenation are complete, but in a hospital laboratory where many hemoglobin determinations are made each day, the actual reading of the samples in the photometer may be delayed.

That the time of reading of the diluted sample is important became apparent to us when we attempted to standardize several makes of photoelectric instruments. It was found on certain occasions that the light absorptive power of the solutions decreased on standing at room temperature. In Table I is shown the variation in hemoglobin values of two blood samples by the carbonate method, the diluted blood being kept at room temperature.

TABLE I. DECREASE IN HEMOGLOBIN VALUES OF BLOOD SAMPLES DILUTED 1:200 IN 0.1 PER CENT CARBONATE AND KEPT AT ROOM TEMPERATURE

TIME IN MINUTES	GRAMS HEMOGLOBIN* PER 100 C.C. BLOOD	
	SAMPLE 1	SAMPLE 2
2	14.0	13.0
12	13.0	12.3
20	12.8	11.6
32	12.1	11.2
37	11.6	10.8
47	11.1	10.5

\*Hemoglobin values were read from a standard curve used in connection with a Coleman Model 6. Transmission values were read with the instrument set at 540 mμ. The standard curve relating oxyhemoglobin concentration to transmission values was prepared from suitable dilutions of blood standardized with a Van Slyke manometric apparatus.

Other samples of blood were studied in this manner and it was found that some showed greater variation, while some were quite stable. The principal variable in this work appeared to be temperature.

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To test this possibility, a blood after dilution in carbonate, which had been stable for 90 minutes at room temperature ( $26^{\circ}\text{C}.$ ), was immersed in a water bath at  $40^{\circ}\text{C}.$  In 15 minutes the hemoglobin value dropped 0.9 Gm. and after 30 minutes, 2.0 Gm. per 100 c.c. blood. Although  $40^{\circ}\text{C}.$  is above our laboratory room temperature, we found that during the course of our work the temperature had varied from  $26$  to  $37^{\circ}\text{C}.$  The greatest instability in oxyhemoglobin values was found to have occurred on the days when the temperature had been  $37^{\circ}\text{C}.$  Although these studies had been made on the Coleman Junior Clinical Spectrophotometer Model 6,\* the same phenomenon (oxyhemoglobin instability at  $40^{\circ}\text{C}.$ ) is easily demonstrated on filter photometers.

It was observed that as the oxyhemoglobin solutions decreased in light absorptive capacity (measured at 540  $\mu$ ), the solutions changed from the bright red color characteristic of oxyhemoglobin to a dull brownish-red color. This color change was investigated spectrophotometrically, by determining the absorption curves of the pigments present in a blood diluted with carbonate and kept at  $40^{\circ}\text{C}.$

The oxyhemoglobin content of the blood sample by the Van Slyke manometric method was 14.25 Gm. per 100 c.c. A Cenco-Sheard spectrophotometer with 1 cm. cells was used for these studies, the entrance slit being 1 mm. and the exit slit 2.5  $\mu$ .

Dilution of the blood was accomplished by adding 0.1 c.c. blood to 20 c.c. freshly prepared 0.1 per cent sodium carbonate† in a 50 c.c. flask. The flask was stoppered, shaken, and then immersed to one-half its depth in a constant temperature water bath at  $40^{\circ}\text{C}.$  Samples of the diluted blood were withdrawn at intervals, cooled to  $26^{\circ}\text{C}.$  and absorption data determined in the Cenco instrument. In Fig. 1 are shown the absorption curves of the pigments present at 0, 60, and 180 minutes and at 24 hours.

The 0-hour curve shows the two, well-defined maxima of oxyhemoglobin at 541 to 542  $\mu$  and 576  $\mu$ . The 1-hour curve indicates that a considerable change has occurred in the oxyhemoglobin molecule, for K at 576  $\mu$  decreased from 0.625 to 0.369, and the K value for the 541  $\mu$  maximum dropped from 0.595 to 0.430. The absorption curve of the 3-hour sample shows a rather broad maximum in the 540  $\mu$  region, but the 576 maximum has disappeared. The 24-hour absorption curve shows the complete disappearance of specific absorption maxima in the 540 to 576 region.

Since it was apparent that the oxyhemoglobin was being converted to a pigment (or pigments) with different absorption characteristics, an attempt was made to determine the nature of such substances.

After the 0-hour sample had been used to obtain the data for the curve on Fig. 1, it was treated with a few small crystals of  $\text{K}_3\text{Fe}(\text{CN})_6$ ,<sup>3, 4</sup> to convert the hemoglobin to methemoglobin. The absorption curve of this material is also shown in Fig. 1. Since the absorption spectra of methemoglobin vary greatly with changes in hydrogen-ion concentration,<sup>1</sup> it is necessary to control the pH value. We believe that by converting the oxyhemoglobin in the 0-hour sample to methemoglobin, while other factors are held constant, it is then possible to use such a system for purposes of comparison. A comparison of the curve at 1 hour with the methemoglobin curve suggests that at this time there has been a conversion of most of the oxyhemoglobin to methemoglobin. This is suggested by the lowering of the 540  $\mu$  maximum, and by a still greater lowering of the 576  $\mu$  maximum, to values approximating those of pure methemoglobin.

\*Coleman Electric Co., Maywood, Ill.

†Mallinckrodt's analytical reagent grade sodium carbonate was used throughout this work.

The absorption curve of the pigments at 3 hours, on the other hand, indicates that during this period another change has occurred. The maximum at 540 to 542  $\mu$  has been lowered below that of the known methemoglobin and has been shifted to the left to the 535 to 540  $\mu$  region. The flat curve of the 24-hour sample indicates that a pigment (or pigments) other than oxyhemoglobin and methemoglobin is present. The shape of this curve is somewhat similar to that of hematin in sodium hydroxide.<sup>1, p. 122</sup>

Assuming that the chief pigment in the solution at 3 hours was methemoglobin, the presence in this solution of small amounts of the pigment responsible for the low, flat absorption curve of the sample at 24 hours may explain the difference between the curve of the 3-hour sample and the methemoglobin curve.

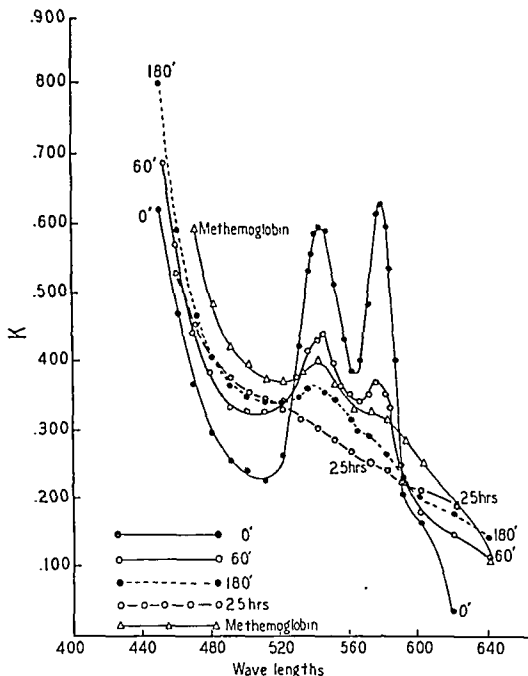


Fig. 1.—Absorption curves of pigments present in a solution of blood diluted 1:200 in 0.1 per cent sodium carbonate and maintained at 40° C. for the indicated periods of time. The ordinate,  $K$ , is defined as:  $K = \frac{n}{1} = \frac{-\log_{10} I}{1}$ . A Cenco-Sheard spectrophotometer with 1 cm. cells was used, the entrance slit was 1.0 mm. and the exit slit 2.5  $\mu$ .

Heilmeyer<sup>1</sup> and Martini and Schuler<sup>5</sup> pointed out the swift spontaneous conversion of oxyhemoglobin to methemoglobin. The latter workers showed that irradiation, especially by short wave lengths, accelerated this conversion. Drabkin and Austin<sup>4</sup> found that blood diluted 1:100 in 0.1N  $\text{NH}_4\text{OH}$  showed some change upon standing at 25° C. but at a 1:1000 dilution in this solvent, absorption values at 540  $\mu$  decreased to one-half in 200 minutes. However, these authors showed that if 5 mm. phosphate at a pH of 7.4 was used as the solvent,

the oxyhemoglobin remained quite constant as measured by absorption values at 540, 560, and 575 m $\mu$ .

From our observations and those of Drabkin, it appeared that the high pH (10.5) of the carbonate solvent, the increased temperature (40° C.), and the dilution of 1:200 all contributed to the instability of the oxyhemoglobin solution.

Since it is difficult to maintain a constant temperature bath at 25 to 28° C. throughout the year, we felt that adequate control of pH might enhance the stability of the solutions being studied. Solutions of blood in distilled water may give a desirable pH value, but the low electrolyte concentration so obtained would favor the precipitation of globulins and thus cause a turbid solution. Preliminary experiments indicated that solutions of oxyhemoglobin between pH values of 6 and 8 were stable. Since lower hydrogen-ion concentrations favor the formation of oxyhemoglobin, the pH level of 8.0 was chosen for further study.

In order to have an adequate electrolyte concentration and, in addition, to have a known hydrogen-ion concentration, it was felt that a dilute buffer solution was indicated. Such a buffer must, however, be dilute enough to permit the laking of red cells in a reasonable time.

The buffer solution we have found most suitable is a mixture of 947 c.c. of M/15 Na<sub>2</sub>HPO<sub>4</sub> and 53 c.c. M/15 KH<sub>2</sub>PO<sub>4</sub>.<sup>6</sup> This buffer can be used in two ways. We have for the past year laked 0.1 c.c. blood in 15 c.c. distilled water, and after hemolysis is complete (2 to 3 minutes at 25 to 30° C.) 5 c.c. of the buffer are added. Since this involves two solutions we attempted to find the minimal concentration of buffer that might be incorporated in the laking fluid. It was found that a 1:20 dilution of the stock buffer permitted the use of 20 c.c. of this solution as the laking fluid.

The recent commercial production of tablets for the preparation of buffers suggested the use of such material for the buffer solution. The Certified Buffer Tablets\* for a pH of 8.0 are composed of sodium tetraborate and potassium acid phosphate. Instructions that accompany such tablets call for the dilution of the tablet in 100 c.c. of warm distilled water. Such a buffer solution does not permit cell hemolysis. However, if a 1:20 dilution of the concentrated buffer solution is made, 20 c.c. of this dilute buffer may be used as the hemoglobin solvent.

In Table II is shown the relative effectiveness of carbonate, diluted M/15 buffer, and the diluted commercial tablets in maintaining the constancy of oxyhemoglobin when the samples were kept at 40° C. for the indicated times. This

TABLE II. HEMOGLOBIN VALUES OBTAINED BY DILUTING 0.1 C.C. SAMPLES OF A BLOOD SPECIMEN WITH THREE SOLVENTS: (1) 0.1 PER CENT SODIUM CARBONATE, (2) DILUTED M/15 PHOSPHATE BUFFER, AND (3) BUFFER TABLET IN 2,000 C.C. H<sub>2</sub>O; SAMPLES KEPT AT 40° C. FOR THE INDICATED PERIODS OF TIME

TIME (MIN.)	0.1 PER CENT CARBONATE	DILUTED PHOSPHATE BUFFER	DILUTED BUFFER TABLET
0	12.9	12.9	12.9
15	12.6	12.9	12.9
30	12.5	12.9	12.9
45	12.1	12.9	12.9
60	11.8	12.9	12.9
75	11.7	12.9	12.9
90	11.5	12.9	12.9
105	11.3	12.9	12.9
120	11.1	12.9	12.9
270	9.8	12.9	12.9

\*Supplied through the courtesy of the Coleman Electric Company, Maywood, Ill.

is merely a group of representative data which are comparable to many similar experiments.

Similar experiments where 0.1 c.c. blood was laked with 15 c.c. water, 5 c.c. of the M/15 buffer subsequently being added, gave results comparable to the dilute phosphate and tablet buffer solutions listed in Table II.

#### CONCLUSIONS

1. Blood diluted 1:200 in 0.1 per cent carbonate, having a pH of 10.5, is unstable at elevated temperatures. Data are presented which indicate that the oxyhemoglobin is probably converted first to methemoglobin and later to hematin derivative.

2. If the pH of the solvent is lowered to approximately 8.0, the oxyhemoglobin solutions are stable over a prolonged period.

Appreciation is expressed to Miss Emily Flanagan for her help in this work.

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## THE DETERMINATION OF THE FREE AND TOTAL CHOLESTEROL OF PLASMA WITH THE PHOTOELECTRIC COLORIMETER

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**I**N UNDERTAKING a prolonged and intensive study of chronic liver disease, it was desired to include among our tests of liver function the estimation of the free and total cholesterol of the plasma. Sperry<sup>1,2</sup> has demonstrated that, when a reliable method of determination is employed, the percentage or ratio of free to total cholesterol is maintained at an almost constant value in the normal adult serum or plasma and that relatively slight variations from this value are indicative of some abnormality in cholesterol metabolism. Since the magnitude of the variation which is significant may therefore be small, it is essential to employ a technique which invariably yields uniform results. The method presented here has proved satisfactory and reliable in our hands, and with the present wide-

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spread use of the photoelectric colorimeter, it was thought that other laboratories might find it equally useful.

In 1934, Schoenheimer and Sperry<sup>3</sup> published a method for the determination of free and combined cholesterol which was based on an initial precipitation as the digitonide followed by the application of the familiar Liebermann-Burchard reaction to its colorimetric estimation. Their procedure gave more uniform results than had heretofore been obtained. The method presented by us is essentially a modification of that of Schoenheimer and Sperry. Certain features which have been incorporated were suggested by Sperry in a later publication<sup>4</sup> and by Alber and Bryant.<sup>5</sup> Data obtained from thirty-five normal adults agree well with those obtained by Sperry.<sup>1</sup> In addition, a prolonged study of chronic liver disease has enabled us to gain evidence for the constancy of results obtainable by this method when applied repeatedly to the same individual over a long period of time.

#### METHOD

The method is described for the simultaneous determination of the cholesterol partition in two or more samples of plasma.

##### *Reagents.*—

1. Acetone-absolute alcohol (1:1)
2. Acetone-ether (1:2)
3. Ethyl ether (reagent grade)
4. Glacial acetic acid
5. 0.2 per cent digitonin in 50 per cent ethyl alcohol\*
6. 100 per cent KOH in distilled water
7. 1 per cent phenolphthalein in 95 per cent ethyl alcohol
8. 5 per cent HCl solution
9. Acetic anhydride (reagent grade†)
10. Concentrated  $H_2SO_4$  (reagent grade)

##### *Procedure.*—

*Extraction of Plasma or Serum:* For each 2 c.c. of plasma or serum, about 40 c.c. of acetone-alcohol are placed in a 50 c.c. volumetric flask and the plasma or serum pipetted into this, drop by drop, with constant shaking. Additional acetone-alcohol is added to just below the calibration mark on each flask and extraction allowed to take place overnight at room temperature. These unfiltered extracts are quite stable and may be kept for some days if necessary. After extraction, the flasks are made up to volume and the contents well mixed and filtered through lipid-free filter paper. Evaporation is avoided by keeping the funnels covered and working rapidly.

*Precipitation of Free Cholesterol:* Ten cubic centimeters of each filtrate are pipetted into 30 c.c. conical centrifuge tubes, 4 c.c. of the digitonin solution added to each, and the mixture stirred well. The rods are left in the tubes which are placed in one or more covered Mason jars, containing a layer of sand, and left overnight at room temperature. The rods are then removed to a numbered rack or nail after both rods and walls of the tubes have been washed down with acetone to free the precipitates. Care must be taken to return each rod to the correct tube in order to avoid the loss of any precipitate which may have adhered to it. The tubes are then centrifuged for twenty minutes at high speed, after

\*Hoffman-La Roche, Inc., Nutley, N. J., or Merck & Co., Inc., Rahway, N. J.

†Eastman Kodak Co., Rochester, N. Y.

which the supernatant fluid is removed with a fine capillary pipette and suction. The stirring rods are replaced in the tubes; rods and tube walls are washed down with 2 to 4 c.c. of acetone-ether. After thorough stirring, the rods are removed and washed down with a small amount of acetone-ether. The tubes are centrifuged at high speed for ten minutes, the supernatant fluid removed as before, and this washing repeated two more times, each time with plain ether. After the final washing, the tubes are placed in a water bath at 40° C. for a few minutes to dry, after which the rods are replaced. Tubes can be left at this stage for some days if kept covered in Mason jars.

*Precipitation of Total Cholesterol:* Five cubic centimeter aliquots of the original filtrates are pipetted into 30 c.c. conical centrifuge tubes which are either graduated or have been marked to contain 9 c.c. A fresh mixture of one part 100 per cent aqueous KOH and three parts absolute alcohol is made up and to each tube are added fifteen drops of this alcoholic KOH. The tubes are well stirred and placed in Mason jars which contain a layer of sand and which have been heated previously to 40° C. The jars are covered tightly and placed in an incubator at 37 to 40° C. for from three to four hours for saponification. Following this, the tubes are cooled and acetone-alcohol is added to the 9 c.c. mark. This is best done with a dropper, washing down the sides of the tubes. One drop of phenolphthalein solution is added to each tube and titrations carried out with 5 per cent HCl. To insure a slight excess of acid, one to two drops are added after the color change. Four cubic centimeters of digitonin solution are added to each, the mixtures well stirred, and the tubes placed in covered jars and allowed to stand overnight at room temperature. From this point, centrifugation and washing are carried out as for free cholesterol.

*Precipitation of Standard Cholesterol:* In our experience, it has been found best to prepare a standard cholesterol curve as follows: Standard cholesterol solutions containing 0.2, 0.4, and 0.6 mg. of cholesterol in 10 c.c. of acetone-alcohol are carried through the determination exactly as for free cholesterol. Initially, it was thought wise to do this with each set of determinations, but as we gained experience with the method, this was found to be unnecessary. It is certainly to be recommended whenever new reagents are prepared or when other possible variables are introduced.

*Development and Reading of Color:* The tubes are removed from the jars and the jars then placed in a constant temperature oven and heated to from 100 to 110° C. The tubes are replaced in the hot jars and left in the oven for one-half hour. The jars are removed from the oven at the end of this time and the precipitates dissolved in glacial acetic acid as follows: 2 c.c. of the acid are added to each, being careful to wash down the walls of the tubes and the stirring rods. The precipitates are stirred well into the solvent and the tubes replaced in the hot sand in the jars for about one minute. The tubes are then placed in a water bath at about 60° C. until complete solution is effected. After solution of all of the precipitates, the tubes are transferred to a water bath at 25° C. While temperature equilibration is being attained, a fresh mixture of acetic anhydride-sulfuric acid is made up in proportions of 40 volumes of acetic anhydride to 1 volume of concentrated sulfuric acid. A sufficient quantity is prepared to permit the addition of 8 c.c. to each tube. This reagent is stable for no longer than one hour at room temperature. To the first tube are added 8 c.c. of the acetic anhydride-sulfuric acid mixture, the contents well stirred, and the tube immediately placed in a water bath at 25° C. in the dark. The remaining tubes are treated similarly, the time of the addition of reagent to the first tube



and the approximate interval between tubes being noted. A reagent blank of 2 c.c. of glacial acetic acid and 8 c.c. of acetic anhydride-sulfuric acid is prepared at the same time. At approximately twenty-five minutes after the addition of reagent to the first tube, the contents of the tubes are transferred to photoelectric colorimeter tubes, protecting the solutions at all times from exposure to light. Colorimeter readings are made on the tubes in the same sequence in which the final reagent was added and are begun thirty minutes after the addition of this reagent to the first tube. After each has been read, readings are repeated in the same sequence until maximal color intensity has been obtained and the reading is stable. We employ an Evelyn photoelectric colorimeter with a light filter having maximal transmission at 660 mu.

*Calculation of Results:* A standard curve is prepared, as noted, from readings obtained on known cholesterol solutions run through the method as for free cholesterol. Color intensity (in units appropriate to the type of colorimeter employed) is plotted against milligrams of cholesterol. That portion of the curve corresponding to a concentration of 0.2 to 0.6 mg. of cholesterol yields satisfactory results with the Evelyn colorimeter and the 660 light filter. The amount of cholesterol present in the aliquot of filtrate used is then read off directly from this curve.

#### RESULTS

*Accuracy.*—To determine the completeness of recovery from pure cholesterol solutions by this method, standard solutions of cholesterol in acetone-alcohol were treated as for free cholesterol and the resultant readings referred to a curve obtained simultaneously from solutions of pure cholesterol dissolved in glacial acetic acid. In Table I are given the results of an experiment of this type.

To check the accuracy of recovery of cholesterol added to plasma, a known quantity of cholesterol in acetone-alcohol was transferred to a 50 c.c. volumetric flask, additional acetone-alcohol added, and 2 c.c. of plasma pipetted into this solution. A separate aliquot of the same plasma was extracted without added cholesterol. The two extracts were carried through the method as described. In the experiments as performed, 1 mg. of cholesterol was added to the extraction flasks, which is equivalent to increasing the plasma values by 50 mg. per cent. Results so obtained with three different plasmas are given in Table II.

TABLE I

CHOLESTEROL DISSOLVED IN ACETONE-ALCOHOL (MG.)	CHOLESTEROL RECOVERED (MG.)	PER CENT RECOVERY
0.200	0.198	99.0
0.300	0.291	97.0
0.400	0.385	96.0
0.500	0.483	98.6
0.600	0.596	99.3

TABLE II

	PLASMA CHOLESTEROL (MG. %)	PLASMA PLUS 50 MG. % CALCULATED (MG. %)	CHOLESTEROL DETERMINED (MG. %)	PER CENT RECOVERY
A.	Free 67 Total 225	117 275	117 276	100.4 100.4
B.	Free 67 Total 219	117 269	115 271	98.3 100.7
C.	Free 34 Total 126	84 175.5	85 173	101.2 98.6

*Plasma Cholesterol Partitions of Normal Adults.*—In Table III are recorded the values for the free, total, and free in total cholesterol obtained by the application of this method to the plasmas of thirty-five normal adult human beings of both sexes, ranging in age from 18 to 63 years. These values agree quite well with those reported by Sperry,<sup>1</sup> who reports a mean value for free in total cholesterol of 26.9 per cent with a standard deviation of  $\pm 1.4$ .

TABLE III. PLASMA CHOLESTEROL—NORMAL ADULTS

SUBJECT	FREE CHOLESTEROL (MG. %)	TOTAL CHOLESTEROL (MG. %)	FREE IN TOTAL CHOLESTEROL (%)
1	79	300	26
2	31	120	26
3	73	250	29
4	30	123	24
5	40	153	26
6	84	288	29
7	46	188	24
8	37	167	22
9	59	217	27
10	38	150	25
11	50	200	25
12	41	157	26
13	69	250	28
14	43	192	22
15	54	200	27
16	45	185	24
17	68	263	26
18	51	183	28
19	31	137	23
20	53	197	27
21	46	170	27
22	49	173	28
23	45	171	26
24	56	217	26
25	36	167	22
26	71	262	27
27	45	175	26
28	66	230	29
29	46	180	26
30	56	198	28
31	37	145	26
32	38	161	24
33	77	262	29
34	61	218	28
35	50	189	26

Mean, 26.1.

Standard deviation,  $\pm 1.9$ .

Coefficient of variation, 7.3 per cent.

*Constancy of the Partition.*—Finally, in Charts I and II evidence is presented for the constancy with which a given individual maintains the percentage of free in total cholesterol over an extended period of time regardless of the total cholesterol values. The subjects used for this experiment were patients with cirrhosis of the liver on the wards of the Third (New York University) Medical Division of Bellevue Hospital. Chart I is compiled from figures obtained from a patient who, over a period of twenty-six months, has shown some clinical response to the treatment of cirrhosis, but none of whose laboratory data gives evidence of significant improvement in liver function. This patient might be said to be maintained in an approximately stationary condition, and it should be noted that all of the values for free in total cholesterol are abnormal. Chart II represents data from a patient who responded well to the treatment of cirrhosis both clinically and as judged by laboratory data. In this case the figures obtained after the second month of observation show a normal percentage of free in total cholesterol.

The results show clearly that although there may be considerable variation in total cholesterol, the percentage of free in total can be maintained within a remarkably narrow range, the level of which appears to correlate well with the clinical status of the patient suffering from cirrhosis of the liver.

CHART I - P.G.

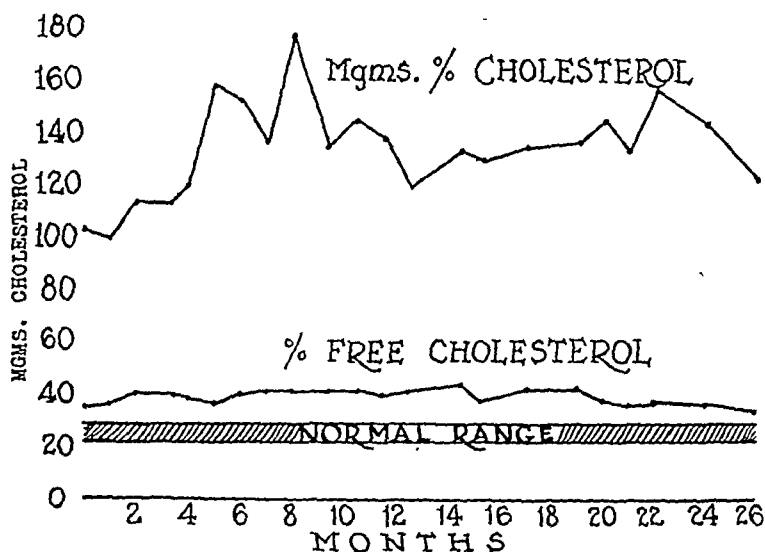
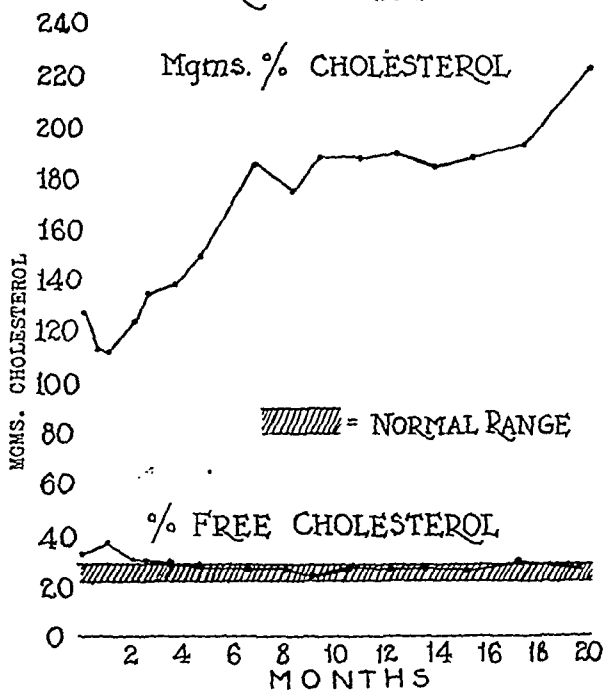


CHART II - W.W.



#### DISCUSSION

Since this method is presented as a modification of that of Schoenheimer and Sperry, discussion will, in the main, be limited to a consideration of the differences between our technique and the original.

*Reagents.*—The use of a 0.2 per cent digitonin solution in 50 per cent ethyl alcohol, as suggested by Alber and Bryant,<sup>5</sup> has proved satisfactory and is much simpler to prepare than the aqueous reagent of Schoenheimer and Sperry, since the digitonin dissolves fairly readily in the alcohol medium. -

Shapiro, Lerner, and Posen<sup>6</sup> proposed the use of a fresh mixture of acetic anhydride and sulfuric acid instead of the separate addition of the two reagents. This departure leads to greater uniformity of results since it avoids the undesirable evolution of heat in the presence of cholesterol, where temperature control is important and also eliminates the difficulties inherent in the accurate measurement of a small volume of the acid. Sperry and Brand<sup>7</sup> has apparently come to the same conclusion.

*Amounts Employed.*—The volumes of plasma, extracts, and reagents given in our method are dependent upon the sensitivity of the photoelectric colorimeter employed and upon the final volume necessary with this colorimeter. These can undoubtedly be modified according to the instrument to be used.

*Extraction of Plasma.*—We have found it unnecessary to use heat in the extraction of plasma if the plasma-acetone-alcohol mixture is allowed to stand overnight at room temperature.

*Total Cholesterol.*—The time periods for saponification and for precipitation of total cholesterol are considerably longer than those employed by Schoenheimer and Sperry. We have not investigated the possibility of shortening either, since these fit in well with the schedule for the entire determination and since completion of both processes is thus ensured.

*Reading of Color.*—It should be noted that, according to our method, repeated readings of an entire series are taken until the readings become maximal and stable. We have been unable to find a single time interval which will give uniform results. One explanation for this is that the time required for maximal color development, under our conditions, varies with the amount of cholesterol present. Also, since it is well established that temperature has a marked effect upon the rate of color development, it is probable that slight uncontrolled day-to-day variations in this factor have influenced our results despite efforts at control. However, repeated readings, even of a large series, are easily taken and the results so obtained with standard solutions yield values of a gratifying constancy, so that we feel this to be a satisfactory point of departure.

*Standard Curve.*—Our standard curve is prepared from cholesterol precipitated as the digitonide prior to color development. In our experience, the values obtained from such a curve are subject to much less variation than those obtained from free cholesterol dissolved directly in glacial acetic acid. In fact, after familiarity with the method is gained, it is found that the digitonide curve is capable of quite accurate day-to-day reduplication.

#### SUMMARY

1. The details are given of a modification of the Schoenheimer-Sperry method for the determination of free and total cholesterol in plasma or serum.
2. The results of the analyses of thirty-five normal human plasmas by this method are presented.
3. The constancy at which an individual can maintain the percentage of free to total cholesterol in his plasma is demonstrated by two extended studies on patients with cirrhosis of the liver.

4. The major points of departure of this method from the original are discussed.

We wish to express our indebtedness to Dr. Charles Hoagland, of the Rockefeller Institute for Medical Research, for the use of some of the normal figures obtained by him with our method.

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### SIMPLIFICATION OF U. S. P. XII MICROBIOLOGICAL VITAMIN ASSAYS

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THE methods for the microbiologic assays of nicotinic acid and riboflavin, outlined in the first bound supplement of the U. S. P. XII, follow procedures widely disseminated in the literature.

Much of the basic work was done by Snell and Strong<sup>1</sup> in assaying riboflavin, Snell and Wright<sup>2</sup> in determining nicotinic acid, and Landy and Dicken<sup>3</sup> in devising a method for the assay of six B vitamins.

The above procedures, as well as those of the U. S. P. XII,<sup>4</sup> are based on the production, by the test microorganism, of acids in series of tubes of basal media containing varying amounts of unknown samples. Acidities are determined by titration with 0.1 N alkali, and potencies are derived by plotting against a standard curve constructed from acidities obtained from dilutions of the standard vitamin in the basal medium. The detailed methods of assay for nicotinic acid and riboflavin, used throughout the following experiments, are very competently described in the first bound supplement of the U. S. P. XII. The only deviation in our procedure in the nicotinic acid assay was to employ thirteen tubes in each standard series and eight tubes in each unknown series as contrasted to the U. S. P. XII requirement of eleven and four in the respective series of known and unknown.

Titration may be made colorimetrically with bromthymol blue as the indicator or electrometrically with a glass electrode. The latter method, using a Beckman Laboratory Model G potentiometer with a system outlined by McQuarrie and Konen,<sup>5</sup> in which glass electrodes and burettes are in an ingenious arrangement, is to be preferred and is used routinely in this laboratory.

Pederson and Bagg<sup>6</sup> point out that many factors affect the relationship of titratable acidity and the final hydrogen-ion concentration in the growth of lactic acid bacteria. The buffer content of the medium and the proportional amounts of acids, especially lactic and acetic acids, that are produced during growth are most important. The varying amounts of lactic and acetic acids elaborated are especially dependent on the type of sugar incorporated in the medium and on the particular species of lactic acid bacteria used as the test organism. It is also noteworthy that when lactic acid is the primary acid a higher hydrogen-ion concentration results than when acetic acid is produced in quantity.

The observations of Pederson and Bagg are most important in the selection of a medium and of a test organism but, once that is accomplished, the necessity for titration in the vitamin assays is superfluous. Since the control standard is identical to the unknown sample, with the exception of the concentration of the vitamin being tested, the relationship between titratable acidity and hydrogen-ion concentration, under the conditions of test, should be constant. Consequently, substitution of a curve of pH values for titration values should suffice.

To verify this contention, data from sixty-eight nicotinic acid and twenty-four riboflavin determinations have been accumulated in which all samples were set up as in the U. S. P. XII methods, and potency determinations were made by plotting both pH and titration values.

The samples assayed included C. T. nicotinamide, C. T. nicotinic acid, solutions of nicotinic acid and riboflavin, yeast extracts, Plebex elixir, and Plebex tablets.

*Nicotinic Acid.*—The data in Table I are examples of results that have been obtained in the assay of nicotinic acid.

TABLE I

NICOTINIC ACID SAMPLE	NUMBER	(A) U. S. P. XII ACID TITRA- TION METHOD	(B) pH METHOD	PER CENT DEVIATION OF (B) FROM (A)
C. T. nicotinamide	435304	57.8 mg.	57.1 mg.	-1.2
C. T. nicotinic acid	441324	27.75 mg.	26.5 mg.	-4.4
Nicotinic acid (ampule)	1340237	2,140 mg.	2,160 mg.	+0.9
Yeast extract dried	91313	3,920 mg.	3,920 mg.	0
Plebex elixir	440039	435 mg.	430 mg.	-1.0
Plebex tablets	441857	1,040 mg.	1,040 mg.	0
Of 68 nicotinic assays				
39 assays average				-2.8
29 assays average				+2.0

TABLE II. TITRATION AND pH VALUES FOR NICOTINIC ACID STANDARD

TUBE	NICOTINIC ACID μg	pH*		ML. 0.1 N ACID†	
		A	B	A	B
1	0.00	5.59	5.60	0.91	1.07
2	0.05	5.12	5.13	2.44	2.34
3	0.10	4.89	4.85	3.56	3.66
4	0.15	4.71	4.71	4.78	4.78
5	0.20	4.59	4.56	5.74	5.69
6	0.25	4.51	4.45	6.15	6.51
7	0.30	4.43	4.43	6.97	6.97
8	0.35	4.39	4.35	7.47	7.42
9	0.40	4.31	4.32	8.09	7.68
10	0.45	4.29	4.26	8.39	8.44
11	0.50	4.28	4.23	8.85	8.80
12	0.00‡	6.66	6.66	-	-
13	0.00‡	6.66	6.67	-	-

\*See Fig. 1.

†See Fig. 2.

‡Uninoculated blanks.

In Table II are included the pH values and acid volumes produced by standard nicotinic acid. These data are plotted in the corresponding Figs. 1 and 2. To facilitate accurate plotting and reading, the curves were drawn on Keuffel and Esser graph paper, No. 358-14. Figs. 1 and 2 were employed in the computation in Table III of the nicotinic acid content of Plebex tablets No. 441857, which were coated tablets containing Anheuser-Busch yeast extract powder with added thiamine HCl and riboflavin.

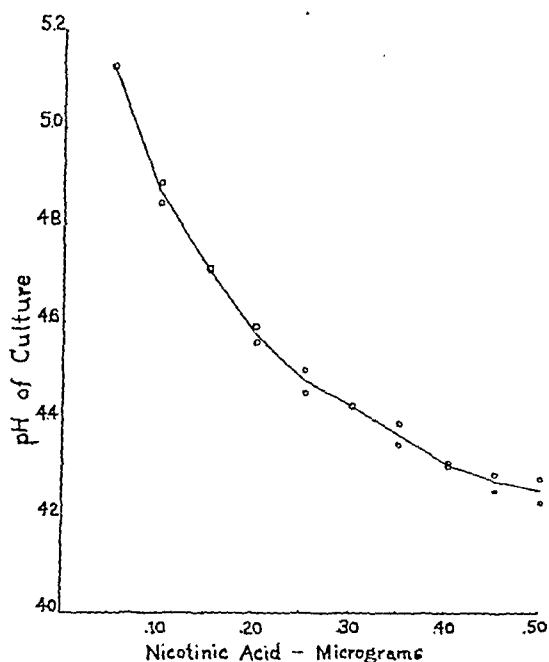


Fig. 1.—Standard nicotinic acid curve plotting pH values of cultures.

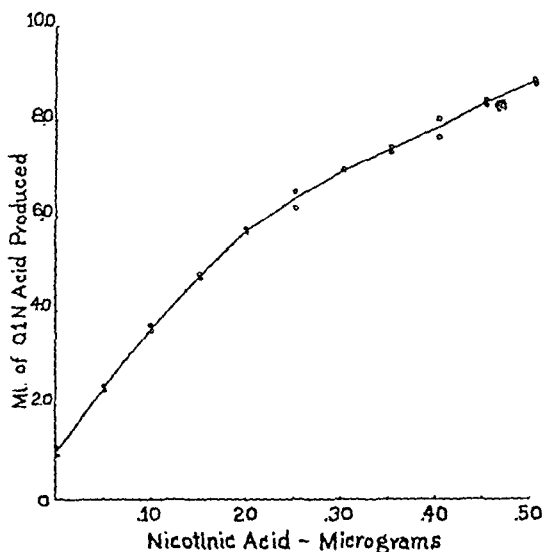


Fig. 2.—Standard nicotine acid curve plotting titratable acid of cultures.

Thirteen of the sixty-eight nicotinic acid assays were also checked by a turbidimetric method. Additional series were set up following U. S. P. XII procedure but, instead of titrating, the turbidity of each was determined with a

TABLE III. COMPUTATION OF NICOTINIC ACID CONTENT OF PLEBEX TABLET No. 441857 BY pH AND ACID TITRATION METHODS

TUBE	ML. DILUTED SAMPLE*	pH		μG. PER CULTURE†		μG PER ML. DILUTED SAMPLE		ML. 0.1 N ACID		μG PER CULTURE‡		μG PER ML. DILUTED SAMPLE		
		A	B	A	B	A	B	A	B	A	B	A	B	
1	1.0	4.89	4.88	.096	.098	.096	.098	3.56	3.66	.098	.102	.098	.102	
2	1.5	4.71	4.69	.150	.157	.100	.105	4.63	4.83	.143	.153	.095	.102	
3	2.0	4.52	4.52	.228	.228	.114	.114	5.14	5.34	.170	.182	—	—	
4	2.5	4.48	4.45	.249	.278	.100	.111	6.36	6.66	.253	.277	.100	.111	
5	3.0	4.41	4.41	.307	.307	.102	.102	7.17	7.17	.322	.322	.107	.107	
6	3.5	4.36	4.35	.360	.377	.103	.108	7.63	7.63	.370	.370	.106	.106	
7	4.0	4.31	4.32	.400	.400	.100	.100	7.78	7.73	.386	.382	.097	.096	
8	5.0	4.24	—§	—	—	—	—	—	—	—	—	—	—	
				Mx		.102	.106					Mx	.100	.107
				Mx A & B		.104						Mx A & B	.104	
.104 × 10,000 (dilution factor) = 1,040 μg      .101 × 10,000 (dilution factor) = 1,010 μg														

\*Dilution of Sample 1-10,000.

†Data from Fig. 1.

‡Data from Fig. 2.

§Off curve.

Klett-Summerson colorimeter. Seven assays showed an average deviation of -3.4 per cent from the U. S. P. XII assay, while six showed an average deviation of +3.4 per cent. Turbidimetric assay data have not been included because of the obvious advantages of the pH method.

**Riboflavin.**—Of twenty-four assays for riboflavin, determined by both U. S. P. XII and pH methods, six pH assays averaged a -0.6 per cent deviation

TABLE IV. TITRATION AND pH VALUES FOR RIBOFLAVIN STANDARD

TUBE	RIBOFLAVIN (μG)	pH*		ML. 0.1 N ACID†	
		A	B	A	B
1	0.00	5.96	6.03	0.82	0.40
2	0.05	5.13	5.13	2.28	2.34
3	0.10	4.82	4.82	3.87	3.92
4	0.15	4.59	4.56	5.39	5.54
5	0.20	4.40	4.38	6.71	7.07
6	0.25	4.20	4.19	8.69	8.69
7	0.30	4.12	4.12	9.25	9.30
8	0.50	4.03	4.03	—	—
9	0.00‡	6.62	6.63	—	—
10	0.00‡	6.63	6.67	—	—

\*See Fig. 3.

†See Fig. 4.

‡Uninoculated blanks.

TABLE V. COMPUTATION OF RIBOFLAVIN CONTENT OF PLEBEX TABLET No. 441857 BY pH AND ACID TITRATION METHODS

TUBE	ML. DILUTED SAMPLE*	pH		μG. PER CULTURE†		μG PER ML. DILUTED SAMPLE		ML. 0.1 N ACID		μG PER CULTURE‡		μG PER ML. DILUTED SAMPLE															
		A	B	A	B	A	B	A	B	A	B	A	B														
1	0.5	5.11	5.10	.053	.054	.106	.108	2.39	2.44	.052	.053	.104	.106														
2	1.0	4.80	4.80	.104	.104	.104	.104	3.87	3.92	.099	.100	.099	.100														
3	1.5	4.51	4.61	.167	.143	.101	.095	5.79	5.04	.162	.137	.108	.091														
4	2.0	4.32	4.40	.218	.196	.109	.098	7.43	6.82	.215	.197	.108	.099														
5	2.5	4.21	4.17	.246	.260	—	—	8.44	9.15	.244	—	—	—														
6	3.0	—§	—	—	—	—	—	—	—	—	—	—	—														
7	3.5	—	—	—	—	—	—	—	—	—	—	—	—														
8	4.0	—	—	—	—	—	—	—	—	—	—	—	—														
				Mx		.105	.101					Mx	.105	.099													
				Mx A & B		.103						Mx A & B	.102														
.103 × 1500 (dilution factor) = 155 μg														.102 × 1500 (dilution factor) = 153 μg													

\*Dilution of Sample 1-1500.

†Data from Fig. 3.

‡Data from Fig. 4.

§Off curve.



from the U. S. P. XII while eighteen pH assays averaged a +3.5 per cent deviation from the U. S. P. XII results.

In Table IV are listed the pH values and acid volumes produced by standard riboflavin. The corresponding Figs. 3 and 4 were used in the computation in Table V of the riboflavin content of Plebex tablets No. 441857.

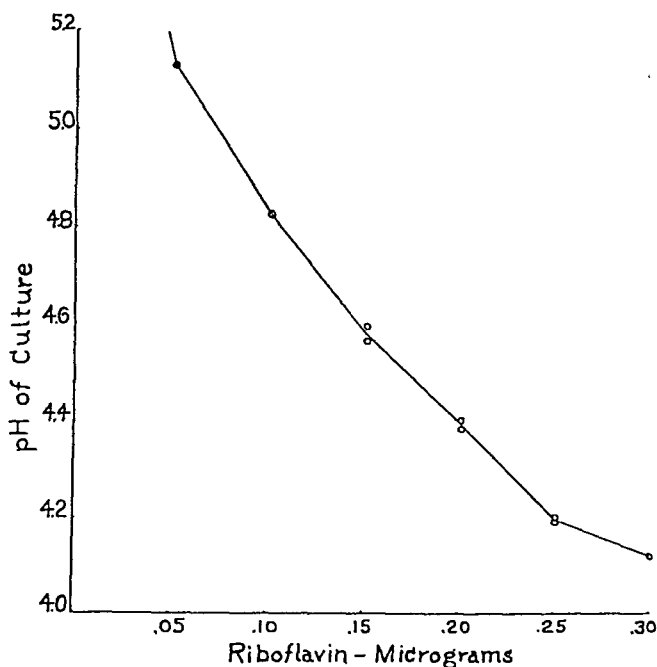


Fig. 3.—Standard riboflavin curve plotting pH values of cultures.

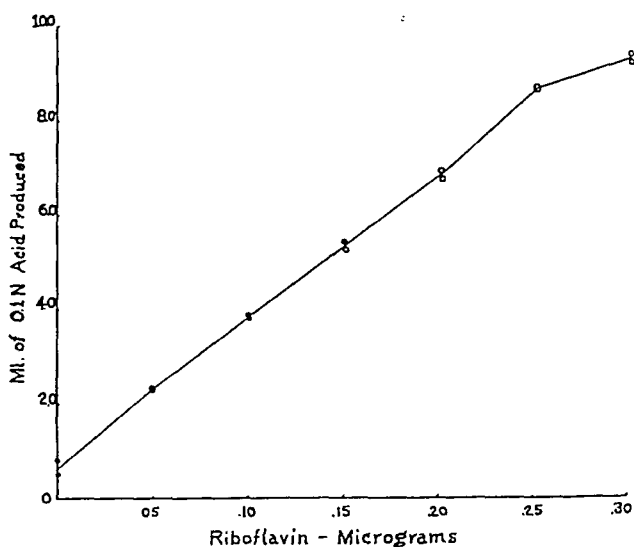


Fig. 4.—Standard riboflavin curve plotting titratable acid of cultures.

As with the nicotinic acid determinations, a small number of U. S. P. XII assays were checked very closely by turbidimetric methods.

Since the actual setup and calculations for all three methods are the

same, any saving in time occurs in the reading of the results. The following comparison shows the superiority of the pH method:

METHOD	TIME REQUIRED FOR READING
U. S. P. XII	45 to 55 minutes for the duplicate standard
	30 to 35 minutes for each duplicate unknown
Turbidimetric	25 to 30 minutes for the duplicate standard
	15 minutes for each duplicate unknown
pH	15 to 20 minutes for duplicate standard
	10 minutes for each duplicate unknown

An obviously additional advantage to the pH method, of course, lies in a minimum of manipulation with, consequently, smaller expected error.

#### CONCLUSIONS

1. It is possible to obtain comparable results in the U. S. P. XII microbiologic vitamin assays by the substitution of pH values for titration values in the construction of the curve for potency determination.

2. Such a procedure has the advantage of requiring less time and manipulation in microbiologic vitamin assays.

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#### AN OPTICAL FLUORESCENCE COMPARATOR

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THE use of atabrine as the principal drug in the treatment of malaria made it highly desirable that simple methods be found to determine the blood level of this drug in large series of patients. The efficiency of the treatment depends essentially on the quick establishment of a satisfactory blood level, and the control of suppressive treatment is only possible if blood levels can be taken at random in forward field hospitals.

The rapid and simple method for the differential diagnosis of bacterial meningitis, as it was recently described by one of us,<sup>1</sup> based upon the estimation of the fluorescein content of the spinal fluid, also requires a simple inexpensive instrument to approximate the fluorescence of the specimens. Many

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tests in clinical vitamin chemistry could be made available to smaller laboratories by the use of such an instrument. In addition, the examination of specimens for their atabrine content can hardly be carried out with a sensitive photoelectric instrument in forward field hospitals in tropical climates since these instruments are not able to stand extreme changes in temperature and humidity as well as violent fluctuations of line voltage. The same holds true for the Dermo-fluorometer,<sup>2</sup> which permits the estimation of atabrine levels in the skin, but which is also sensitive to extreme climatic changes. It seemed desirable, therefore, to design an instrument which would obviate the disadvantages of a photoelectric fluorescence meter without sacrificing too much accuracy.

The instrument described\* permits the estimation of fluorescence by visually comparing the fluorescence of the unknowns with that of a series of fluorescent solutions of known concentration (standards). The optical fluorescence comparator then serves to indicate the place which the unknown takes in a series of these standards.

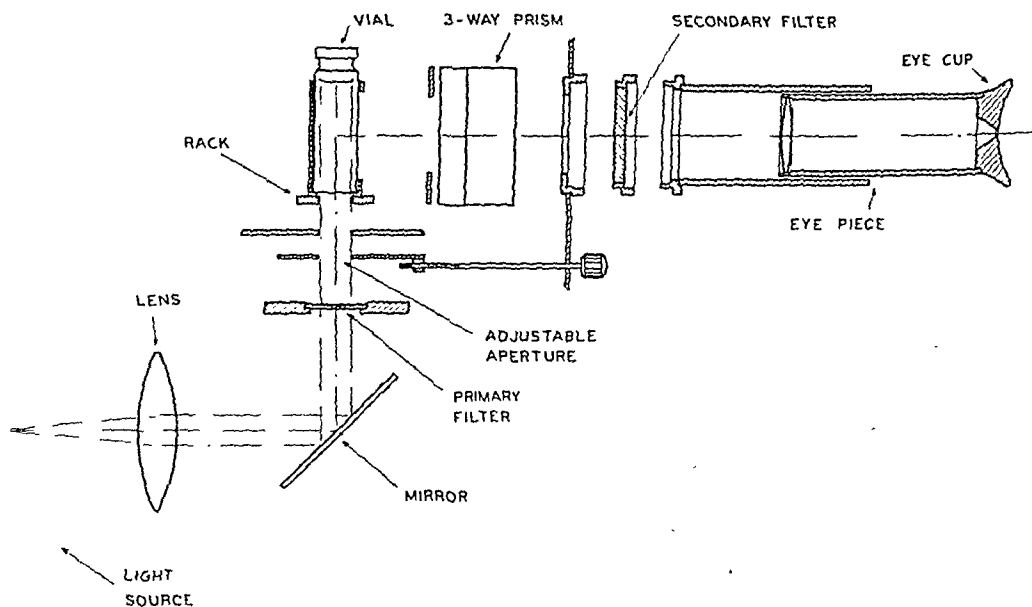


Fig. 1.—Schematic setup of the optical fluorescence comparator.

The light from an incandescent or mercury vapor lamp is collimated by a lens, is deflected by a mirror, and passes vertically through three flat-bottom vials of 5 c.c. content (Fig. 1). The fluorescence thus incited in the liquid is observed horizontally from the front. The optical design is such that the light beam is absolutely parallel and stays so when passing through the solutions in the vials which have ground bottoms. As a result of this, blanks appear perfectly black when viewed through the eyepiece.

A split-field optical-viewing system provides three fields of equal size, directly adjacent to each other (Fig. 2). The three vials brought together into the triple fields are viewed through an adjustable magnifying eyepiece. Primary and secondary filters can be introduced between light source and sample and sample and eyepiece, respectively. The device is constructed in a way that it

\*Developed in cooperation with the Photovolt Corporation, New York, N. Y.

can be used with incandescent or a mercury vapor bulb, depending on what range of wave length of exciting light is desired. Using the incandescent bulb and a primary filter of Corning glass No. 5113 and a secondary filter of Corning glass No. 3480, 0.1 gamma per cent of atabrine extracted by the method of

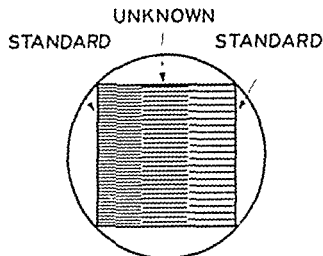


Fig. 2.—Appearance of the three samples through the eyepiece.

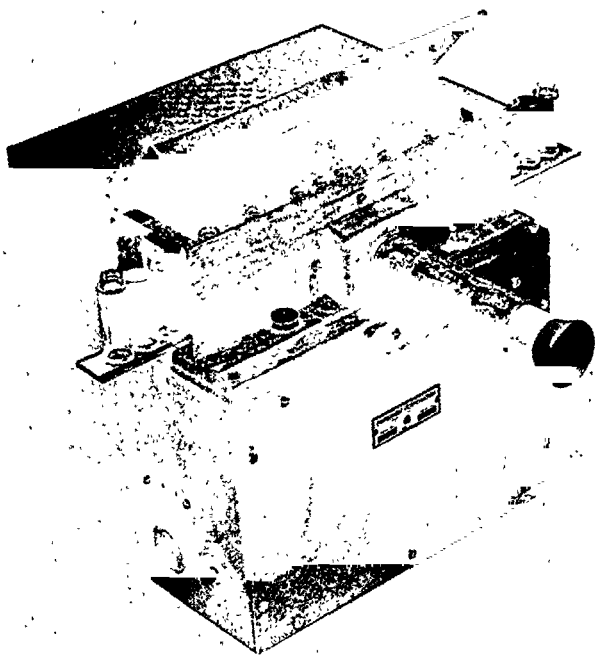


Fig. 3.—The optical fluorescence comparator.

Lange and Matzner can still be clearly recognized. It is possible that this sensitivity for atabrine can still further be improved by using substances that enhance the fluorescence, as described by Brodie and Udenfriend<sup>3</sup> and Ayer-

bach and Eckert.<sup>4</sup> While the accuracy in determinations with a photoelectric fluorescence meter is obviously higher, the lowest detectable amount of a substance seems to be better with the fluorescence comparator, using the human eye as a registering element.

The operation of the instrument is simple and can be carried out by a person with little experience and irrespective of climate and variations in line voltage. The fluorescent standards of known concentration are poured into vials containing 5 c.c. each, and these are inserted in the order of increasing concentration into the sliding rack so that a space is left open between each two. The vial with the unknown is inserted into the space between two standards, and the three vials are then viewed, the two standards occupying the outside fields and the unknown the center field. If the brightness of the unknown is found not to be equal to one of the standards, or to be of intermediate fluorescence, the rack containing the standards is shifted and the unknown inserted into the next gap. This step is repeated until it is found that the unknown is either equal to one of the standards or is between two.

In order to lengthen the life of the exciting lamp, a powerful blower cools the bulb. Both are turned on only during the time of actual measurements by pressing a push button. Three adjustable apertures are provided under the vials to equalize the brightness of the field. This adjustment may only be necessary when the bulb is exchanged. Three vials are filled with the same fluorescent liquid and the knobs (Fig. 3) controlling the apertures are moved until the brightness is even. Once the three fields are equalized in this manner, readjustment will not be necessary, except after installing a new lamp.

#### SUMMARY

A visual fluorescence comparator is described which permits exact determinations of fluorescence of biologic materials as atabrine extracts or fluorescein plasma and spinal fluid samples. The device operates under all climatic conditions and is independent of fluctuations in line voltage.

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# A METHOD FOR DETERMINING THE COAGULATION TIME USING CAPILLARY BLOOD

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SEVERAL years ago one of us (T. K.) developed a method for determining the coagulation time using capillary blood. The current emphasis on changes in the coagulation time following the administration of heparin<sup>1,2</sup> prompts us to publish the method at this time inasmuch as the frequent venepunctures necessary to follow the effect of heparin by the standard Lee-White method complicate the use of this anticoagulant. The inaccuracy of the commonly used capillary tube method<sup>3,4</sup> because of the variable end point and inadequate temperature control is generally recognized.

## METHOD

Thick-walled capillary tubes (40 by 5 mm.) with a 1 mm. bore and ground to a tapered point are fitted at one end with a short length of rubber tubing sealed with a glass plug (Fig. 1, A). After preliminary cleansing with alcohol,

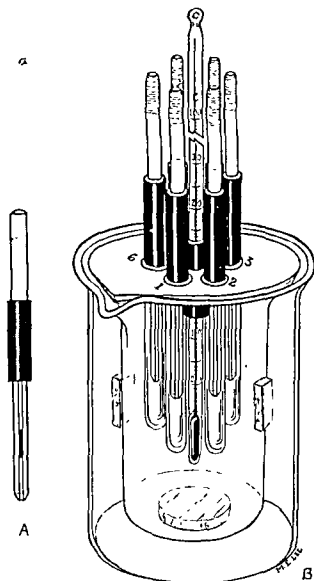


Fig. 1.—A, Capillary tube 40 by 5 mm. with a 1 mm. bore fitted with rubber tubing and glass plug as used in this method. B, Large empty beaker with an inner smaller beaker and fitted with a lid containing holes for six small, flared test tubes and a thermometer. Cork insulation is used to separate the inner and outer beakers. Cutler sedimentation tubes have proved satisfactory for the inner air jacket tubes.

From the Department of Physiology and Pharmacology of the School of Medicine of the University of Pittsburgh

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the palmar surface of the distal phalanx is punctured, with the point of a No. 11 scalpel blade. This blade produces a free-flowing drop with a minimum of trauma and therefore little tissue fluid admixture. Squeezing the finger to obtain a drop of blood must be avoided because a decrease in the coagulation time from thirty seconds to one minute may be induced. A 1 cm. column of blood is drawn up to the midportion of the capillary tube by compressing and releasing the rubber tubing and partially removing the glass plug. Two samples are quickly taken from the same wound and placed immediately in a water bath at 25° C. At one-half minute intervals (or oftener as one approaches the coagulation time) the column of blood is displaced a short distance by compressing the rubber tubing. The end point is taken as that time when the column of blood cannot be displaced without leaving a definite stain on the glass wall. The average of the samples is taken as the coagulation time.

Two details must be observed if duplicate results are to be obtained: (1) the tubes must be uniformly clean and dry; (2) the temperature must be carefully controlled. To prevent the coagulated blood in the tubes from drying, immediately after using, they are placed in hydrogen peroxide in the vertical position. To clean the tubes thoroughly they are rinsed in forcefully running water, placed overnight in sulfuric acid-chromate cleaning solution, rinsed thoroughly, and air dried. If even soft wire is used to remove clot from the lumen of the tube it should be carefully done to avoid scratching the glass. To keep the temperature of the tubes at 25° C., a large empty beaker with an inner smaller beaker filled with water at 25° C. and fitted with a lid containing holes for six small test tubes and a thermometer has proved satisfactory (Fig. 1, B). Temperature control is necessary as a change from 20 to 28° C. may alter the coagulation time as much as 1.5 minutes. It is advisable to have the coagulation tubes stand in the water bath for from ten to fifteen minutes before using. The thick-walled glass permits the relatively small volume of blood to take on the temperature of the glass. A temperature of 25° C. has

TABLE I. SUCCESSIVE COAGULATION TIMES USING THE CAPILLARY TUBE METHOD DESCRIBED\*

SUB- JECT	TEST 1				TEST 2				SUB- JECT	TEST 1				TEST 2			
	TUBE 1		TUBE 2		TUBE 1		TUBE 2			TUBE 1		TUBE 2		TUBE 1		TUBE 2	
	MIN.	SEC.	MIN.	SEC.	MIN.	SEC.	MIN.	SEC.		MIN.	SEC.	MIN.	SEC.	MIN.	SEC.	MIN.	SEC.
1	2	15	2	15	2	35	2	20	26	3	00	3	00	3	15	3	45
2	2	30	2	30	2	30	2	45	27	4	15	4	15	4	30	4	15
3	3	30	3	25	3	15	3	30	28	5	00	4	30	5	00	4	45
4	3	45	4	00	4	00	3	50	29	4	15	4	30	4	30	4	15
5	4	00	4	30	4	15	4	45	30	4	00	4	10	4	00	4	15
6	4	30	4	15	4	15	4	15	31	3	30	3	45	4	00	3	30
7	4	00	4	20	4	30	4	30	32	4	00	4	00	4	15	4	30
8	4	25	4	15	4	30	4	30	33	5	00	5	15	5	15	5	00
9	3	20	3	30	3	30	3	15	34	5	30	5	30	5	30	5	30
10	3	45	4	00	4	00	4	10	35	5	00	4	45	4	45	4	30
11	4	00	4	15	4	10	3	50	36	4	30	4	45	4	15	4	30
12	3	30	3	30	3	30	3	30	37	5	30	5	15	5	15	5	15
13	4	00	4	20	4	30	4	30	38	3	30	3	15	3	15	3	00
14	3	20	3	00	3	10	3	10	39	2	45	2	30	2	50	2	40
15	5	00	5	00	4	45	4	45	40	2	15	2	30	2	00	2	15
16	4	10	4	00	4	15	4	20	41	4	00	4	10	3	45	4	00
17	3	00	3	10	3	20	3	00	42	3	40	3	15	3	45	3	40
18	4	10	4	00	4	00	3	50	43	4	10	4	30	4	30	4	15
19	4	30	4	30	4	30	4	15	44	4	00	4	00	4	20	4	40
20	3	50	4	00	4	00	3	45	45	3	45	4	00	4	00	4	30
21	3	30	3	45	3	15	3	45	46	4	10	4	05	3	45	3	30
22	4	10	4	30	4	30	4	30	47	2	45	3	10	2	40	3	00
23	4	00	3	45	3	45	3	45	48	4	10	3	50	3	45	4	10
24	2	25	2	45	2	45	2	45	49	3	20	3	30	3	00	3	20
25	3	40	3	50	3	20	3	20	50	3	45	3	50	3	45	3	30

\*Each test was made in duplicate.

been chosen because it approximates room temperature and can therefore be readily maintained.

## RESULTS

In Table I are given the results of tests performed on fifty normal individuals. Each test was done in duplicate and was immediately repeated. Note that with one exception (Subject 44) the greatest variation in the coagulation time of consecutive samples from any one individual was thirty seconds or less. The shortest coagulation time in this series was two minutes, fifteen seconds, and the longest five minutes, thirty seconds at 25° C. Because of the reproducibility of the method, it is felt that these variations represent true differences in the coagulation time of these normal people. Both the median and the average of the coagulation time in this series were close to four minutes. In Table II are compared the results obtained by this method with the standard Lee-White venepuncture procedure. The Lee-White method records the time necessary for relatively complete clotting to occur, whereas this method, because of the delicacy of the end point, indicates the time necessary for the initiation of coagulation. This undoubtedly explains the difference in normal coagulation times between the Lee-White and this method. In our experience the prolonged coagulation times observed after the administration of heparin also may be readily obtained by this method.

TABLE II. COMPARISON OF THE CAPILLARY TUBE METHOD WITH THE LEE-WHITE PROCEDURE\*

SUBJECT	CAPILLARY TUBE						LEE-WHITE		
	TUBE 1		TUBE 2		AVERAGE		TUBE 1 MIN.	TUBE 2 MIN.	TUBE 3 MIN.
	MIN.	SEC.	MIN.	SEC.	MIN.	SEC.			
1	4	10	4	45	4	28	8	11	13
2	4	10	4	25	4	17	9	10	12
3	2	45	3	00	2	52	10	12	13
4	4	10	3	45	4	27	10	11	12
5	3	20	3	30	3	25	10	12	13
6	3	45	3	50	3	48	10	11	12
7	4	10	4	00	4	05	12	12	13
8	3	30	3	40	3	35	11	12	13
9	4	05	4	20	4	12	10	12	13
10	3	00	3	20	3	10	9	11	12

\*Tubes 1 and 2 in the Lee-White column included only for completeness, column 3 being the coagulation time as indicated by that method. As noted in the text, this capillary tube method indicates the initiation of coagulation, whereas the Lee-White method indicates relatively complete coagulation.

## SUMMARY

A simple method of determining the coagulation time using capillary blood is presented; normal values with this method ranged from two minutes, fifteen seconds to five minutes, thirty seconds at 25° C. with a variation in duplicate tests of thirty seconds or less. Both the median and the average coagulation time in this series were very close to four minutes.

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# A SIMPLE PROCEDURE FOR DETERMINING THE APPROXIMATE CONCENTRATION OF PEPSIN IN GASTRIC CONTENTS

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NEW YORK, N. Y.

THE method to be described is a simplification of the procedure described by Barowsky, Tauber, and Kleiner.<sup>1</sup> The same basic principle is utilized, namely, the milk-clotting action of gastric fluid at pH 5.0. There is no rennin in human gastric juice; at any rate, not in that of the adult.<sup>2</sup> Therefore, a measure of the milk-clotting power is a measure of peptic activity.

The difference between the procedure mentioned and the present one is that in the former the greatest dilution of gastric juice which produces a clot is determined, while in this modification the length of time required for a single tube to clot is ascertained. The other, and more accurate procedure, requires the preparation of ten or more dilutions. The objection to this is the time consumed in preparing these dilutions, particularly if great accuracy is not required. Only one dilution and one portion of buffered milk are used in the simplified method. The length of time required for clotting in this single test varies inversely with the amount of pepsin present (Table I).

TABLE I. INTERPRETATION OF RESULTS

CLOTTING TIME (MIN.)	TENTATIVE COMPARISON WITH NORMAL	APPROXIMATE NUMBER OF UNITS/C.C. GASTRIC JUICE	SUGGESTED METHOD OF REPORTING
Less than 3	Very high	Over 2,500	++++
3 to 4	Moderately high	2,001-2,500	+++
4 to 10	Usual normal	1,001-2,000	++
10 to 15	Low	501-1,000	+
Over 15	None to trace	0-500	0 or trace

## *Solutions Required.—*

A. Buffer. Dissolve 42 Gm. of NaOH in about 500 c.c. distilled water; add 115 c.c. 80 per cent acetic acid; dilute to 1,000 c.c. This keeps well.

B. Fresh cow's milk.

Equal volumes of A and B are mixed well; 10 c.c. are needed for each test.

## *Procedure.—*

1. Centrifuge or filter gastric contents, if necessary.

2. Test reaction with Congo red paper.

3. (a) If acid, dilute 1 c.c. to 50 c.c. with water. Mix well.

(b) If not acid, dilute a small quantity accurately with an equal volume of 0.1 N HCl; mix well and then dilute 1 c.c. to 25 c.c. with water. Mix again.

4. Place a test tube containing 10 c.c. of buffered milk in water at 20° C.

From the Department of Physiology and Biochemistry, New York Medical College, Flower and Fifth Avenue Hospitals.

Demonstrated at the Graduate Fortnight, New York Academy of Medicine, Oct. 11-22, 1943.

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5. Add 1 c.c. of the diluted gastric fluid to the test tube of milk, mix quickly, replace in the water bath, and note the time.

6. Note the time when the milk clots (i.e., either thickens on tilting the tube or small white particles separate out).

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## BOOK REVIEWS

**Essentials of Allergy.** By *Leo H. Cripp, M.D.*, Assistant Professor of Medicine and Lecturer in Immunology, School of Medicine, University of Pittsburgh; Diplomate in Internal Medicine (1937); Fellow of the American Academy of Allergy; Chairman Department of Medicine, Montefiore Hospital and Senior Staff in Medicine, Presbyterian and Passavant Hospitals, Pittsburgh; Consultant in Allergy to the Medical Service of the United States Veterans Administration. J. B. Lippincott Company, Philadelphia. Price \$5.00. Cloth with 381 pages and 42 illustrations.

**Contents:** 1. Hypersensitiveness. 2. Anaphylaxis. 3. Allergy. 4. Diagnosis of allergy. 5. Treatment of allergy. 6. Pollen allergy (Hay fever). 7. Bronchial asthma. 8. Nasal allergy. 9. Skin allergy (Allergic dermatoses). 10. Serum allergy. 11. Drug allergy. 12. Bacterial allergy. 13. Fungus allergy. 14. Physical allergy. 15. Miscellaneous allergy. 16. Allergy in children. 17. Diagnostic skin tests.

The author has succeeded in condensing into a small volume a great deal of the present-day knowledge of *clinical allergy*. The book contains much useful information for undergraduate medical students and practicing physicians, for whom it is primarily intended. The information is, in general, authoritative, based upon modern clinical standards in a rapidly developing field. Numerous case reports are presented. The subject matter is concise and presented without controversial discussion. The thoughtful, analytical reader, however, will find several minor inaccuracies, together with several misleading and confusing statements. The following are called to attention:

1. On the inside of the front cover anaphylaxis is defined as "a state of hypersensitiveness produced experimentally in animals by injecting them for the second time with the same protein." (On page 2 we find that by "animals" is meant only "a lower animal"). In reality the "state of hypersensitiveness" is frequently, and indeed usually, produced by the *first* injection. It is the reaction or shock which is produced by the *second* (or subsequent) injection.

2. The author has followed the custom, which is prevalent in certain quarters, of applying the term "anaphylaxis" to the hypersensitiveness produced in guinea pigs, rabbits, dogs, etc., by injections of certain foreign substances (e.g., sera). But when these same substances are injected into another species of animal, namely, man, the resulting hypersensitiveness is called "allergy." This, the reviewer believes, is an artificial and misleading separation of analogous (if not identical) phenomena. The issue is further confused by the classification of "serum allergy" as item IIB under "acquired allergy" as distinct from item A-anaphylaxis, yet on page 260 we find that in ordinary serum sickness "precipitins last for several days to a few weeks . . ." and "anaphylactic antibodies usually accompany them."

3. The terms "acquired" and "familial" are used in a confusing manner. There may be, and in fact there is, a familial tendency to acquire certain allergies. But the allergy is, in fact, acquired at some time during the life of the individual—usually after birth, occasionally during intrauterine life. No allergy has been proved to be other than "acquired."

4. On page 15, paragraph 2, there appears the following statement: "There is no evidence to indicate that it is possible to sensitize human beings artificially regardless of whether they are normal or atopic." This sentence is incorrect as stated and is, in fact, contradicted in the following paragraph in which it is admitted that injections of horse serum may result in skin sensitivity and even in the development of atopic reagins.

5. On page 84 may be found recommendations for strengths of extracts used for intradermal testing, including fish and eggs in "1-10 dilution." The reviewer considers this strength decidedly unsafe (unless preceded by scratch tests).

6. In the discussion of "atopic or familial (allergic) dermatitis" beginning on page 226, the statement is made that the allergen is brought to the skin by the hematogenous route and is usually a food although it may be an inhalant. The shock tissues are said to

be the endothelial cells lining the skin capillaries, and the diagnostic test is given as the scratch or intradermal test. While this is probably the prevailing view among allergists, there are some dissenting voices, including that of Dr. R. L. Cooke, who wrote the foreword to the book. These dissenting voices will probably increase greatly in number with the passage of time. These dissenters have reasons for believing that, in this disease, the hematogenous route is not important in the transportation of allergen to the skin, that the allergens are not often foods or inhalants, and that scratch and intradermal tests are not often of diagnostic value. The author, however, probably does well to avoid such controversial issues in a book of this nature.

F. A. S.

**Clinical Roentgenology of the Digestive Tract.** By *Maurice Feldman, M.D.*, Assistant Professor of Gastroenterology, University of Maryland; Assistant in Gastroenterology, Mercy Hospital; Consulting Roentgenologist, Sinai Hospital; Consulting Roentgenologist, Sinai Hospital. Second edition. Williams & Wilkins Company, Baltimore, Md., 1945. Price \$7.00. Cloth with 769 pages.

Dr. Feldman's book will be a very useful reference for students and practitioners. The clinical factors of etiology, incidence of disease, age distributions, symptoms, and pathology are adequately stated for their use in roentgenological interpretation. The descriptions of the roentgenological pictures are concise and full and give differential details. The discussions given of many rare diseases and conditions of the gastrointestinal tract with careful statement of their rarity should be especially valuable to students and casual users of the x-ray in diagnosis. For experienced and specialized gastrointestinal roentgenologists the book gives an informative orientation to the study of the tract and its appendages. Only in reference to the illustrations may they find more than personal differences. More illustrations would be helpful to the less experienced and to beginners in the specialty. The bibliography is not extensive but well selected from world sources and adequately pertinent to the nonreview presentation. The author has very well achieved his object in presenting a general guide to the roentgenological diagnosis of the digestive tract and in indicating its importance and value. The book will be valuable to all who may be interested, either casually or especially, in gastroenterology and should find a place in their library.

**Studies of Burns and Scalds (Reports of the Burns Unit, Royal Infirmary, Glasgow, 1942-1943).** Medical Research Council, Privy Council, Special Report Series No. 249. Price, 4s. Od. net. Hard paper cover with 209 pages.

In this excellent clinical report 127 cases are summarized, thirty in considerable detail. Observations include many chemical studies as well as a variety of clinical data. The data recorded will certainly prove of great interest to those who are studying burns clinically, and many of the observations will be a stimulus to those interested in a few of the many unsolved problems. The local treatment is simple and consists of gentle cleansing with a detergent, followed by the application of a dressing over a sulfonamide cream. Systemic therapy consists of plasma and serum transfusions in large amounts.

An interesting and important experiment was carried out in four patients in whom a green dye was injected intravenously to determine the extent of the increased capillary permeability. It was demonstrated in at least one patient that the center and therefore the more severely burned area remained unstained by the dye. This obviously indicated a type of burn which had destroyed the circulation through the skin and was therefore associated with less extravasation. This observation perhaps is not sufficiently emphasized. It has an important bearing on the general assumption that the amount of fluid lost in a burn and therefore the amount required to correct hemoconcentration varies roughly with the extent of the burn. It seems clear that this would apply only if the entire burned area were associated with extravasation. This would be more likely when the thermal injury did not coagulate the skin, for example, in scalds. Flame burns, on the other hand, might destroy the circulation through the injured area and thus limit the degree of exudation. Such differences may explain some of the discrepancies between the degree of hemoconcentration and the extent of the burn which the authors noted. These considerations emphasize the need for describing the type of burn present in each case. The title of the monograph suggests that the cases would be so classified, but this promise is not fulfilled. It might have been profitable to correlate the degree of hemoconcentration with the kind of thermal injury (that is, scald or burn) as well as with its extent. A very instructive series of photographs shows the rapidity

with which edema of the face subsides spontaneously after a superficial burn. Twenty-four hours after the injury the patient's facial edema, which nine hours before prevented the eyes from opening, had almost completely subsided.

Intravenous injections were carried out rather extensively, the fluid being largely serum or plasma. In some cases concentrated serum was used. The amounts injected were large and by present standards might be called excessive. The indications for the use of intravenous fluids were at first based on the development of hemoconcentration or other evidence of need. Soon this plan was changed to one in which plasma or serum was given early and more or less as a routine in order to *prevent* hemoconcentration. This latter plan was said to be justified by the results, which, however, on analysis seemed equivocal. Indeed, the authors admit that at least one patient had an excessive amount of intravenous fluid. For example, one 6-year-old child with a burn involving but 10 per cent of the body was given intravenously 1,900 c.c. (50 c.c./kg.) of plasma during six hours.

Anemia was observed only in the more serious cases. Red blood cell levels about 50 per cent of normal were reached in practically all of the patients with the most extensive burns on the tenth to the fourteenth day. In an attempt to explain this anemia, studies were made of the fragility of the red cells. Significant alterations were observed in the response of the red cells in burn patients as compared with the fragility of the red cells in normal subjects subjected to the same test. Moreover, many patients showed gross hemolysis in their circulating blood and a few contained hemoglobin in the urine. All patients showing hemolysis in the plasma in large amounts were very extensively and severely burned and all except one died within twelve hours of injury.

The notable contribution of the Glasgow School is the demonstration that infection can be greatly reduced if not almost eliminated by meticulous attention to the fundamental principles of aseptic surgery. Although the authors believe that their use of local sulfonamide therapy was partly responsible, the evidence indicates that the outstanding reason for their successful control of infection was a consistent drive in the handling of the cases against contamination and cross infection. This required painstaking attention to detail, careful masking of the patient and personnel, complete sterilization of everything coming into contact with the burn, and even systematic elimination of air-borne infection from dust in the room, on the floor, and by movement of personnel. It was quite evident from their experience that such detailed though simple measures definitely resulted in a tremendous decrease in the incidence of infection. This experience emphasizes the fundamental fact already demonstrated during World War II, that chemotherapy will not of itself prevent or eliminate infection and that in the case of most surgical wounds, fundamental principles of surgical technique demand precedence and emphasis. Chemotherapy can be effective only as an adjunct or adjuvant, not as a substitute for these principles.

**Introduction to Animal Biology.** By John B. Parker, Ph.D., Professor Emeritus of Biology, Catholic University of America, Washington, D. C.; and John L. Clarke, Ph.D., Assistant Professor of Biology, Retired, Catholic University of America, Washington, D. C. Second Edition. The C. V. Mosby Company, St. Louis, 1945. Price \$3.75. Cloth with 532 pages.

# A PATHOLOGIC STUDY OF TSUTSUGAMUSHI DISEASE (SCRUB TYPHUS) WITH NOTES ON CLINICOPATHOLOGIC CORRELATION

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## INTRODUCTION

THE known rickettsial diseases of man excluding the still moot problem of trench fever fall into four distinct groups: typhus, spotted fever, tsutsugamushi disease, and Q fever. Typhus is carried by lice and fleas, spotted fever by ticks, and tsutsugamushi disease by mites. The rickettsiae of Q fever are found in ticks, but epidemiologic studies are incomplete. Two distinct varieties of typhus, murine and human, are recognized and strain differences, notably in virulence, are found between individual members of the other three groups. The existence of complete cross-immunity between all varieties and strains within each of these groups, however, and the invariable absence of cross-immunity between members of different groups, are ample justification for regarding the classification as definitive.

Scrub typhus has become the commonly employed clinical designation of a rickettsial disease which recently has assumed importance because of its occurrence among troops in New Guinea and in adjacent islands of the Southwest Pacific area. The careful work of Lewthwaite and Savaor<sup>1</sup> has shown that this disease is essentially identical clinically, pathologically, and immunologically with tsutsugamushi disease. The term scrub typhus, therefore, should be regarded as having only temporary value and must eventually be discarded in the interest of nosological clarity, just as the term Sao Paulo typhus has been abandoned in favor of (Brazilian) spotted fever now that the disease is known to be identical with Rocky Mountain spotted fever.

Scrub typhus certainly is no more closely allied to the typhus group than to the spotted fever group of diseases, and there is every reason for separating it sharply from both. This purpose would be achieved most simply by calling it tsutsugamushi disease. Any strain differences which may come to light as a result of study could be indicated by names analogous to murine typhus and human typhus. In this way confusion in terminology and perhaps unwarranted assumptions regarding therapeutic indications could be avoided.

For a general discussion of tsutsugamushi disease, including the clinical picture, diagnosis, and epidemiology, reference is made to recent papers by Alhm and Lipschitz<sup>2</sup> and Farner and Katsampes.<sup>3</sup> These authors express a preference for the name tsutsugamushi disease.

*Historical Orientation.*—Tsutsugamushi disease has been much less thoroughly studied, in its etiologic and pathologic aspects, than have typhus and

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spotted fever. Japanese investigators in 1928<sup>4</sup> and 1931<sup>5</sup> demonstrated the etiologic agent in the testicle of the rabbit and in the anterior chamber of the rabbit's eye, propagated the agent by these rather inconvenient methods of inoculation, and named it *Rickettsia tsutsugamushi* (*orientalis*) (*nipponica*). Lewthwaite and Savor,<sup>6</sup> in a single instance, isolated a strain in guinea pigs rendered vitamin deficient. In the course of recent work, including ours, the isolation and propagation of strains have been accomplished repeatedly by the intraperitoneal injection of human blood into white mice and guinea pigs. This has greatly facilitated the laboratory study of the disease. The repeated isolation of strains in this manner, in contrast to previous failures, can be explained perhaps as a phenomenon of strain variation. The reaction of the guinea pig to these recently isolated strains is apparently identical with that described by Lewthwaite and Savor in their single success.

The histopathology of diseases of the tsutsugamushi group has heretofore been described only briefly.<sup>7-9</sup>

*Objectives of the Present Study.*—Our study is based on an analysis of fifty-five fatal cases of tsutsugamushi disease occurring in American troops in British and Dutch New Guinea and adjacent islands. A considerable number of experimental animals (mice and guinea pigs) reacting to strains isolated from these patients were available also for study. The purposes of the study are (1) to describe in detail the pathologic changes associated with the disease; (2) to compare these changes with those found in fatal cases of typhus and spotted fever; and (3) to determine whether an analysis of these data would bring out pathologic or physiologic differences between tsutsugamushi and the other two groups of rickettsial disease which might act as a guide to clinical research.

#### THE ETIOLOGIC AGENT

Our observations on the etiologic agent of the disease (*R. tsutsugamushi*) will be confined largely to its morphology, since detailed biologic and immunologic studies by others will soon be available.

The direct demonstration of rickettsiae in human tissues has been achieved only in film preparations from pericardial, pleural, and peritoneal fluid. Here they are present in much smaller numbers than in similar preparations from infected mice and guinea pigs and usually are found only after long search. Thus far we have not been successful in finding definite rickettsiae in paraffin sections of human tissues. On the basis of experience with typhus tissues, we have no reason for stating that rickettsiae are more difficult to demonstrate in tsutsugamushi than in typhus material. In spotted fever, however, rickettsiae are somewhat easier to demonstrate.

The presence of rickettsiae in smears from the serous surfaces of mice, guinea pigs, and human beings, their demonstration by Japanese workers in the testicle and in the anterior chamber of the eye,<sup>4, 5</sup> and their cultivation in tissue culture without loss of virulence<sup>10</sup> leave no serious doubt concerning their etiologic significance in tsutsugamushi disease.

Morphologically, *R. tsutsugamushi*, as seen in film preparations from the peritoneum of the mouse, certainly does not appear to differ greatly from *Rickettsia prowazeki* and *Dermacentrolexenus rickettsi*, the agents of typhus and spotted fever, respectively. In all three, the predominating form is a minute diplobacillus. In size, *R. tsutsugamushi* is indistinguishable from *R. prowazeki* and also from *D. rickettsi* in its more common forms.<sup>11</sup> Forms resembling the large lanceolate diplococci, which occasionally are seen in spotted fever, were not

observed by us. We found that bipolar and diphtheroid forms were more common than in either typhus or spotted fever; however, since identical forms have been seen in large numbers in the brain of typhus-infected mice and occasionally in typhus-infected yolk sacs, this observation seems to be of doubtful differential value. Long chains and thread forms were not seen in our material, but such forms are found only under unusual conditions.

Cells packed and distended with hundreds of rickettsiae, so commonly seen in films from the peritoneum of experimental animals reacting to murine typhus or to human typhus, were not found in this study. Individual cells contained from ten to sixty organisms, suggesting the picture seen in smears from experimental spotted fever in guinea pigs, but the organisms tended to form loose clusters rather than being diffusely scattered as in spotted fever.

Spotted fever is characterized by the presence of rickettsiae not only in endothelial cells, but also in smooth muscle cells of arterioles, while in typhus, organisms are seen only in intimal endothelial cells.<sup>11</sup> A comparison of tsutsugamushi with typhus and spotted fever from this point of view cannot be made until suitable material is available.

The presence of rickettsiae in nuclei, a feature of spotted fever but not of typhus, can be studied only in sections and is rarely demonstrable in mammalian tissues.<sup>11</sup> The material in which they can best be demonstrated is the infected chorioalantoic membrane of the fertile egg,<sup>12</sup> which was not available in this study.

#### PATHOLOGY

The general similarity of the pathologic picture to that of typhus and spotted fever<sup>14</sup> became apparent early in the course of this study. In all three diseases, the pathologic changes undoubtedly are direct results of the multiplication of rickettsiae within the endothelial cells or smooth muscle cells of capillaries, arterioles, venules, and less often of larger vessels. Swelling and proliferation of the intimal endothelium, cellular infiltration of the wall of the larger vessels, perivascular accumulation of mononuclear cells, and occasional thrombosis and hemorrhage are the essential features of the reaction. These changes are most noticeable in the skin, brain, and myocardium but are present to some extent in nearly every organ, so that the disease may be characterized as an acute generalized inflammation of the vascular lining, the vascular wall, and the perivascular tissue.

*Gross Observations.*—In general, the gross changes seen post mortem are not striking. The most distinctive finding upon external examination of the body is the local primary lesion at the site of inoculation by the bite of the larval mite. It is a cutaneous ulceration, varying from 2 to 15 mm. in diameter, usually covered with a blackish crust, the so-called eschar, which is only slightly adherent to a shallow grayish-pink, granular crater, with sharply defined margins. The skin surrounding the ulcer is moderately red. In our series, the primary lesion, when secondarily infected by pyogenic organisms, was often enlarged and presented a dirty gray surface with purulent drainage. Not infrequently the ulcer was in a healing stage and relatively inconspicuous. The primary lesion was observed at autopsy in 89 per cent of our cases, occurring in the locations listed in order of frequency: trunk, 32 per cent; axilla, 22 per cent; external genitals, 12 per cent; thigh, 12 per cent; lower leg, 10 per cent; inguinal region, 8 per cent; upper extremity, 2 per cent; neck, 2 per cent. In one case, two typical primary ulcers with eschars were present, one on the abdomen and the other on the thigh.



The *skin rash* observed clinically fades very rapidly after death and was present in only two of our patients upon both of whom autopsy was performed immediately after death on the twelfth day of the disease. The lesions consisted of confluent, red, mottled macules from 1 to 2 cm. in diameter scattered over the trunk, upper arms, and thighs.

Necrotic lesions of the skin were absent and hemorrhagic lesions were most unusual, appearing in but two of our series. On one patient there was an area of purpura 8 cm. in diameter over the sacrum, accompanied by recent petechial hemorrhages over the arms and hands, which appeared terminally on the thirteenth day after an extreme elevation of temperature to 108° F. In the second patient, many recent petechial hemorrhages were scattered over the skin of the chest and face. These also appeared a few hours before death on the thirteenth day of the disease.

The *serous cavities* commonly presented characteristic gross changes. Free fluid was often encountered which was cloudy and straw colored and contained small flecks of whitish-yellow exudate. The serous surfaces tended to be slightly dull and injected, occasionally studded with punctate hemorrhages.

In 56 per cent of our cases the cloudy straw-colored fluid in the pericardial sac was increased, although not enough to cause cardiac embarrassment, as it averaged less than 100 c.c. The petechiae were most frequently located on the posterior surface of the sac.

The pleural cavities presented a similar appearance with free fluid in one or both sacs in 53 per cent of our cases, the amount ranging from 30 to 500 c.c. in 76 per cent, being over 500 c.c. in 24 per cent. In at least one-half of our cases this effusion was not associated with bronchopneumonia, and subsequent microscopic studies made it obvious that the condition represented a rickettsial pleuritis rather than a secondary bacterial infection.

A slight to moderate increase of peritoneal fluid was observed in only one-fifth of our cases.

The *heart* exhibited relatively mild changes and was of normal weight. The myocardium at times was slightly flabby and, in a few cases, contained minute, focal, pale, brownish-gray areas of degeneration or, more rarely, small recent focal hemorrhages. The endocardium was generally smooth, pale, and glistening; petechiae were rare. Valvular damage attributable to rickettsial infection was not seen.

The *aorta, vena cava, and other large vessels* were altogether without gross changes attributable to rickettsial infection. In one case, however, we observed thrombosis of the right adrenal vein and its tributaries and of a branch of the deep circumflex iliac vein associated with massive hemorrhage into the left rectus muscle, the left psoas, and left retroperitoneal space.

The *lungs* varied greatly in appearance. Usually hemorrhage and congestion of the entire organ were the constant and striking features in the cases uncomplicated by secondary pneumonia. Weight increase was moderate to marked, and the tissue was only partially crepitant. The cut surfaces were blotchy dull red to deep purple and exuded copious dark bloody fluid. Small multiple hemorrhagic infarcts occurred in six of our patients. The mucosa of the tracheobronchial tree was deeply injected and the lumen was partially filled with sticky blood-tinged mucus. The pulmonary picture closely resembled that of the hemorrhagic pneumonitis of influenza. Less often congestion and edema were present without the hemorrhagic changes. When secondary bronchopneu-

monia was superimposed, the characteristic picture was partly masked. Microscopic examination in all cases revealed essential changes which will be described later.

The *spleen* was characteristically enlarged, firm and dull, and slaty blue. Flecks of fibrinous exudate were usually scattered over its capsule. The enlargement ranged from 300 to 700 Gm. in 65 per cent of the cases and from 200 to 300 Gm. in 28 per cent. The organ weighed over 700 Gm. in two cases, while in two others the weight was normal. Cut surfaces were deep purplish red; the splenic follicles were not prominent; the pulp was considerably softened. Focal areas of hemorrhage were fairly common and infarets, occasionally multiple and sometimes large, were present in four cases.

*Lymphadenopathy* involving particularly the mesenteric, retroperitoneal, tracheobronchial, axillary, and inguinal groups was observed in approximately two-thirds of our cases. The glands were discrete, mildly to moderately swollen, succulent, and soft; the cut surfaces were pink with occasional areas of focal hemorrhage and necrosis. As a rule the lymph nodes in the region of the primary ulcer were more enlarged and areas of focal hemorrhage and necrosis more frequent. When the ulcer was secondarily infected, the lymph nodes commonly were purulent.

The *liver* had undergone no distinctive gross changes. Mild hepatic enlargement was present in 58 per cent of the series, and small, subcapsular hemorrhages were occasionally seen. The cut surface usually had a dull, cloudy appearance. The biliary tree showed no abnormality.

The *gastrointestinal tract* was without characteristic gross features, although usually punctate mucosal hemorrhages were scattered throughout its entire length. In one instance petechial hemorrhages were countless and a large amount of blood filled the entire tract from stomach to rectum; in rare cases the Peyer's patches were mildly swollen. Small focal hemorrhages were occasionally present in the serosal coats.

The *pancreas* showed no significant changes.

The *suprarenal glands* appeared normal, except in one case in which marked hemorrhage into the parenchyma had resulted from thrombosis of the adrenal vein and its tributaries.

The *kidneys* were usually swollen and congested. On the cut surfaces the dusky red pyramids stood out sharply against the pale opaque cortical parenchyma. Small infarets were present in two cases.

The *bladder* contained occasional small submucosal hemorrhages.

The *testicles* and the *bone marrow* appeared grossly normal.

The *brain* rarely showed distinctive gross changes. The vessels of the leptomeninges were often congested. Mild cerebral edema was occasionally evidenced by slight flattening of the convolutions and gyri, usually with concomitant increase in the brain weight. In a few cases minute pinpoint hemorrhages were apparent on the cut surfaces of the brain, frequently in the brain stem and pons, rarely in the basal ganglia, the cerebrum, and the cerebellum.

The spinal fluid at times was slightly increased in amount, and occasionally was cloudy.

*Microscopic Observations.*—The heart was most seriously involved since the dominant lesion in all cases was acute nonsuppurative myocarditis, focal as well as diffuse, and varying in severity. This inflammatory reaction was patchy in distribution and usually most severe in the interventricular septum and the left ventricle. It was characterized by perivascular infiltration of mononuclear

cells, edema, and cellular infiltration of the larger fibrous septa, and also by a more patchy interstitial edema and cellular infiltration.

The most marked and constant finding was perivascular infiltration of mononuclear cells, chiefly plasma cells with fewer large mononuclear cells, occasional lymphocytes, and polymorphonuclear leucocytes (Fig. 1) and sometimes large multinucleated cells with vesicular nuclei and basophilic cytoplasm. This reaction occurred about vessels of all sizes but more abundantly about the smaller arterioles, venules, and capillaries. Frequently the vascular endothelium was swollen or thickened by proliferation, usually asymmetrically. The intima occasionally presented a frayed-out appearance; leucocytes often adhered to the lining, and more rarely, recent mural thrombi overlay the injured

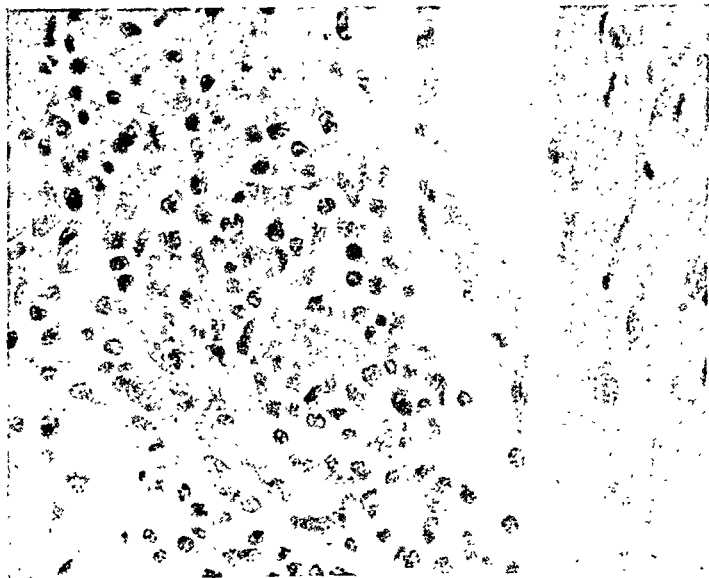


Fig. 1.—Perivascular mononuclear cell infiltration centering around a small damaged vessel in a fibrous septum in the myocardium.



Fig. 2.—Mononuclear cell infiltration in an edematous connective tissue septum in the myocardium.

endothelium. Occasionally the entire vessel wall was loosely infiltrated with mononuclear cells.

The larger connective tissue septa were edematous and infiltrated to a varying degree with mononuclear cells (Fig. 2).

The more diffuse type of myocarditis was characterized by the presence of columns of mononuclear cells, chiefly plasma cells, lying in the connective tissue interstices between individual muscle fibers and in close relationship to capillaries (Figs. 3 and 4). The capillary endothelium often showed swelling and proliferation. Areas of focal hemorrhage were not unusual where this inflammatory reaction was severe. Degenerative changes in the cardiac muscle fibers varied from cloudy swelling, loss of striation, and fatty degeneration to actual necrosis. A resultant early fibroblastic proliferation was occasionally seen.



Fig. 3.—Diffuse myocarditis in tsutsugamushi disease.

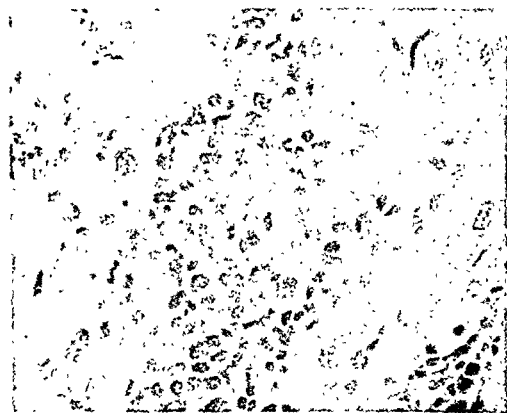


Fig. 4.—Diffuse myocarditis. High power to show nature of the cellular infiltration.

The *endocardium* was mildly edematous and the connective tissue layer slightly infiltrated with mononuclear cells. Occasionally, microscopic hemorrhages were present.

The *epicardium* usually exhibited increased vascularity. In all vessels changes were identical with those described in the myocardium. Focal hemorrhages were common. The tissue was sparsely infiltrated with mononuclear cells and occasional polymorphonuclear leucocytes. The serous endothelium was often swollen and proliferating. In several cases inflammatory changes had taken place in the subendothelial regions, and even in the media of the coronary arteries (Fig. 5).

The *aorta*, throughout its course, exhibited a mild acute aortitis characterized by perivascular infiltration of mononuclear cells about the vasa vasorum.



Fig. 5.—Coronary artery showing endangitis and involvement of the media.



Fig. 6.—Aorta. Note the perivascular infiltration and the focal necrosis in the outer portion of the media.

Occasionally the vascular damage was severe and in two cases was attended by focal necrosis in the media with interruption of the elastic fibers in its outer portion (Fig. 6). Mild infiltration of mononuclear cells, plasma cells, lymphocytes, and histiocytes was occasionally seen in the subendothelial connective tissue of the intima.

The *lungs*, in all cases not obscured by complicating secondary pneumonia, showed an interstitial inflammatory reaction of such constant and typical features as to suggest a rickettsial origin. The arterioles and venules and sometimes the larger vessels of the interlobular septa, usually presented changes of varying degree such as perivascular infiltration of mononuclear cells, endothelial swelling and proliferation, infiltration of the wall, and not infrequently

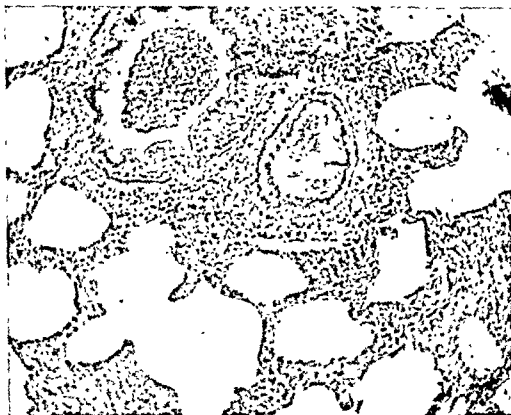


Fig. 7.—Interstitial pneumonitis in tsutsugamushi disease, probably of rickettsial origin. Note the inflammatory cells in the peribronchial regions and in the alveolar walls.

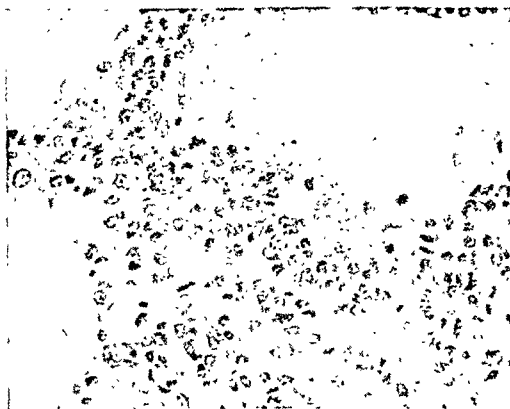


Fig. 8.—Interstitial pneumonitis. High power to show the types of cells present in the alveolar walls.

thrombosis. The interlobular septa were markedly edematous and diffusely infiltrated, chiefly by plasma cells and large mononuclear cells. The alveolar walls were congested and correspondingly infiltrated with mononuclears. The alveolar lining cells were swollen and proliferating. Most air spaces contained pink-staining edema fluid, a few mononuclear cells, and only occasional polymorphonuclear leucocytes. The alveoli contained no purulent exudate; neither did the primary and secondary bronchioles. A hyaline membrane lined the alveolar walls in several cases. Many areas were flooded with frank hemorrhage. These lesions varied considerably in severity and, as in the myocardium, were patchy in distribution. The pulmonary lesions are shown in Figs. 7 and 8. In some cases the basic condition of the lungs was overshadowed by a secondary acute bronchopneumonia and bronchiolitis which presented the usual histologic features.

The *pleura* showed changes similar to those in the epicardium, with edema, vascular and perivascular infiltration of mononuclear cells, endothelial swelling and proliferation, and occasionally thrombosis and hemorrhage. The connective tissue layer often was infiltrated with mononuclears; the serous cells commonly were swollen and proliferating.

The lesions in the *spleen* were those of acute splenitis with marked congestion of the pulp, frequently accompanied by hemorrhage and occasionally by infarction. The red pulp was densely infiltrated with mononuclear cells, plasma cells, large mononuclears, and lymphocytes. In some instances polymorphonuclear neutrophils and eosinophils were present in considerable numbers. Phagocytosis of erythrocytes and cellular debris by reticuloendothelial elements were prominent. The vascular changes were similar to those described in other organs. The Malpighian corpuscles were inconspicuous but often showed central hyaline necrosis.

The splenic capsule was often mildly infiltrated with mononuclear cells and polymorphonuclear leucocytes; the serous cells were swollen and proliferating. At times an exudate of fibrin, desquamated serous cells, and mononuclear cells adhered to the surface.

The *lymph nodes* presented a histologic picture similar to that seen in the spleen, characterized by acute lymphadenitis with congestion and occasionally hemorrhage and focal necrosis. The lymph sinuses were stuffed with large pale mononuclear cells, plasma cells, lymphocytes, and polymorphonuclear leucocytes. The fixed reticuloendothelial cells were prominent and increased in number, often containing phagocytized debris (Fig. 9). Focal hyaline necrosis was present in some follicles. These changes were usually more pronounced in the lymph nodes near the primary ulcer. Lymph nodes draining secondarily infected ulcers showed the usual picture of acute suppurative lymphadenitis.

The *gastrointestinal tract* was without distinctive changes except for vascular lesions of the type found in other organs. Areas of hemorrhage into the mucosa were common. In the case with massive terminal hemorrhage into the gastrointestinal tract, vascular lesions were unusually severe and there were innumerable foci of hemorrhage from the mucosal capillaries into the mucosa.

The mucosa was often infiltrated with considerable numbers of large mononuclear cells.

The *liver* presented no constant histologic features. The hepatic cells showed varying but usually mild degrees of cloudy swelling and fatty degeneration. The Kupffer cells were swollen and prominent; the cytoplasm at times was vacuolated, at times containing engulfed erythrocytes. Numerous mononuclear cells, both plasma cells and large mononuclear cells, were scattered through

the sinusoids. The portal spaces were mildly edematous and infiltrated with plasma cells and large mononuclear cells, with fewer lymphocytes, neutrophils, and eosinophiles. In a few cases there was marked hepatic degeneration with necrosis. The degenerative changes were most marked about the central portion of the lobule, with relatively mild associated inflammatory reaction.

The *pancreas* showed slight microscopic change limited to perivascular infiltration in the supporting connective tissue. In rare instances vasculitis and perivasculitis were more severe and accompanied by edema and mild diffuse infiltration of the loose connective tissue bands sufficient to constitute an acute mild interstitial pancreatitis.

In the *suprarenal glands* clumps of mononuclear cells, chiefly plasma cells and large mononuclear cells, infiltrated the cortex and medulla. The larger vessels in the connective tissue of the central portion of the medulla showed perivascular cellular infiltration and occasionally actual evidence of vascular injury such as endothelial swelling, proliferation, or thrombosis. These changes varied considerably in severity within a given organ as well as from case to case.



Fig. 3.—Mesenteric lymph node. Note the reticuloendothelial hyperplasia.

The *kidneys* showed characteristic focal interstitial lesions and, occasionally, evidence of severe vascular damage and glomerular injury. Usually, focal collections of plasma cells, large mononuclear cells, and lymphocytes, with occasional polymorphonuclear leucocytes, were scattered irregularly through the parenchyma, but they occurred most frequently about the junction of cortex and medulla. At times these collections encompassed two or three disintegrating tubular segments or a glomerulus. The long straight capillaries of the medulla were generally congested and frequently showed perivascular infiltration of mononuclear cells. The vessels throughout the kidney presented changes similar to those in other organs.

The epithelium of the convoluted tubules exhibited cloudy swelling. The tubules sometimes contained albuminous material.

The glomeruli were not uniformly affected; most of them were normally cellular and had well-filled capillaries; others, however, showed a definitely increased cellularity and were ischemic. Occasionally the capsular epithelium had proliferated and formed thin crescents. A few glomerular loops were



frankly necrotic. The glomerular spaces sometimes contained albuminous material, erythrocytes, or epithelial cells. At times pericapsular spaces were infiltrated by mononuclear cells.

Focal hemorrhage, occasionally seen in the parenchyma, was most common in the medulla. Recent anemic infarcts were seldom present in the cortex, but in these cases thrombi could sometimes be demonstrated in damaged arteries.

The *urinary bladder* showed only the vascular changes described, chiefly in the submucosa.

The *testicle* was not seriously affected and exhibited only characteristic vascular lesions, mild interstitial edema, and occasionally thrombosis and hemorrhage. The vascular lesions were much milder than those occurring in the heart, lung, and brain, and no more severe than those seen elsewhere.



Fig. 10.—Subepithelial tissue in the primary cutaneous ulcer. Note the vasculitis and perivascular collections of mononuclear cells.

The *primary cutaneous ulcer* in the earlier phases showed coagulation necrosis of the epidermis and superficial layers of the corium. The characteristic vascular lesions were present in the corium (Fig. 10). Later ulceration occurred and the ulcer base was formed by degenerating leucocytes and cellular debris. The underlying corium was edematous. There was moderate diffuse cellular infiltration, chiefly lymphocytes, especially about the epidermal appendages as well as about nerve bundles; the appearance was that of a localized cellulitis. The inflammatory reaction did not tend to spread laterally but sometimes extended downward into the panniculus. Healing occurred with epithelialization of the surface and absorption of the exudate. Vascular and perivascular infiltration, however, persisted even after surface healing had taken place.

*Skin* from areas which exhibited rash at the time of death showed comparatively mild changes. The capillary loops were moderately congested and surrounded by a few infiltrating cells, chiefly lymphocytes. The superficial vessels of the corium were similarly affected, but the deep vessels showed little change. Thrombosis and hemorrhage were exceedingly rare. The basal layer of the epidermis was mildly edematous.

The *bone marrow* sometimes was rich in plasma cells and large mononuclear cells.

The brain showed considerable variation from case to case. In some cases there was only mild perivascular infiltration of plasma cells and large mononuclear cells about the vessels of the leptomeninges with a few cells scattered through the arachnoid membrane. The arterioles, venules, and capillaries were congested to varying degrees.

In other cases a well-defined acute serous meningitis and an acute focal encephalitis were present. There was frequently marked perivascular infiltration of the vessels of the leptomeninges, and the arachnoid membrane and space were densely infiltrated with large pale mononuclear cells and plasma cells (Fig. 11). The vessels in all parts of the brain showed a similar cellular infiltration into the perivascular sheath, at times dense cuffs were formed. The vessels themselves exhibited the characteristic lesions described. Focal nodules

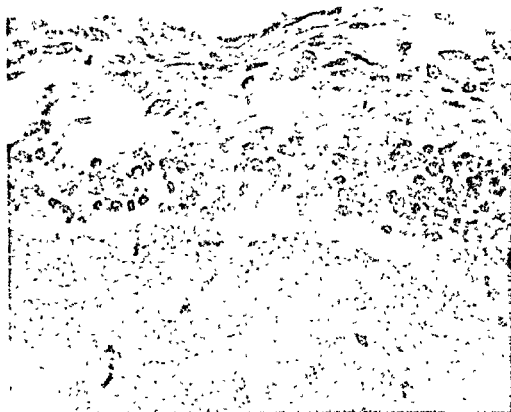


Fig. 11.—Mononuclear cell infiltration in the arachnoid membrane, giving the picture of mild meningitis.

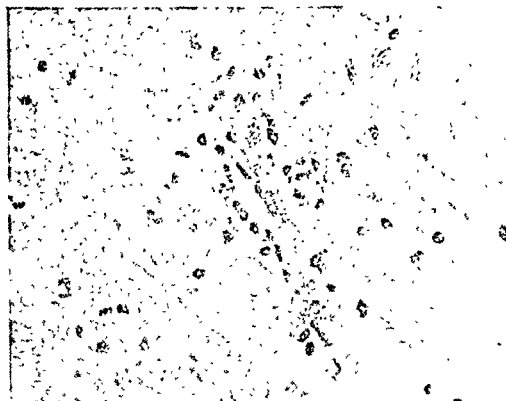


Fig. 12.—A damaged cerebral capillary, with early endothelial proliferation and perivascular reaction.

similar to those seen in typhus<sup>13</sup> and spotted fever<sup>14</sup> were scattered through the brain stem, cerebellum, and cerebrum. These had formed about small vessels or capillaries, probably incidental to vascular damage by rickettsial invasion in the endothelium. The nodules consisted of a loose conglomeration of irregularly arranged glial cells and large mononuclear cells, with occasional polymorphonuclear leucocytes. The surrounding brain tissue was edematous and lesions occasionally encompassed a neuron which showed varying degrees of chromatolysis. These lesions were seen in all stages of formation, from the early vascular type with marked perivascular cellular infiltration and endothelial proliferation, followed by thrombosis or closure of the lumen by endothelial proliferation and swelling, to the final, small, focal, granulomatous nodule (Figs. 12, 13, and 14); serial sections always proved the vascular relation.

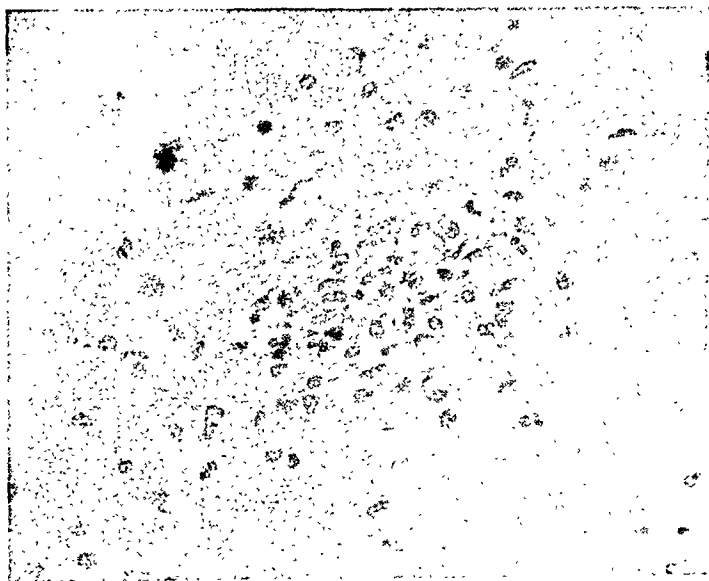


Fig. 13.—Early focal lesion in the cerebellum. Note the presence of several neutrophils.

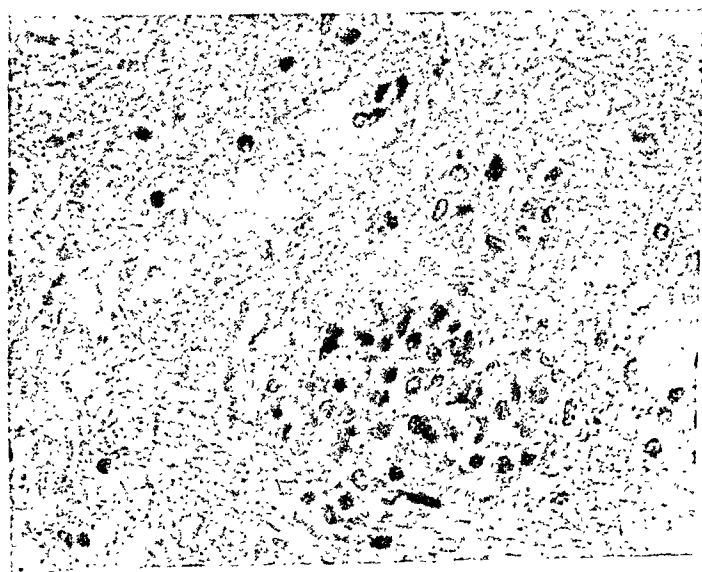


Fig. 14.—Later stage in development of focal brain lesion, with predominance of neuroglial cells.

Nodules were most frequent in the pons and medulla, less frequent in the basal ganglia, cerebellum, and cerebrum and spinal cord. It was unusual to see more than from one to three to a section. Punctate focal hemorrhages were often seen, scattered through brain stem and cortex. These hemorrhages sometimes were around nodules but often were unrelated to them. Nodules were seen much more frequently if death occurred after the twelfth day. In one case in which death occurred on the thirty-third day from pulmonary complications after an intervening afebrile period, the healing stage of the nodule was studied. The nodules were more compact than at earlier stages; plasma cells and large mononuclear cells were inconspicuous; cells with elongated oval nuclei and small glial elements predominated. The inflammatory reaction in the meninges and in vessels of the brain had practically disappeared.

Focal areas of demyelination, which have been considered characteristic of spotted fever,<sup>14</sup> were rarely, if ever, encountered.

#### PATHOGENICITY AND PATHOLOGY IN EXPERIMENTAL ANIMALS

During the course of this study, the opportunity arose to observe the pathogenicity and pathology of tsutsugamushi disease in experimental animals, coincident with transmission studies carried out by other members of the Department of Bacteriology of the Third Medical Laboratory and members of the United States of America Typhus Commission

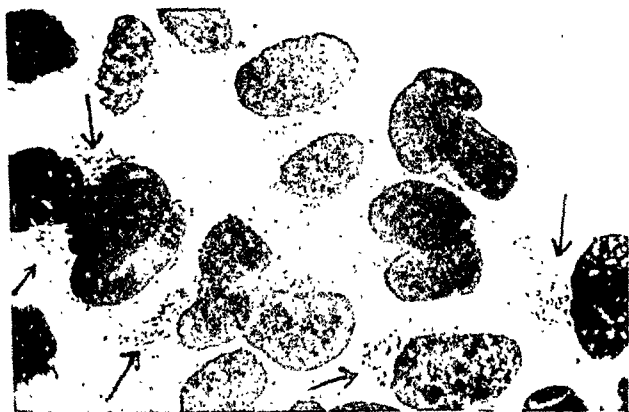


FIG. 15.—Serosal cells from peritoneal cavity of white mouse reacting to tsutsugamushi disease. Arrows point to loose collections of rickettsiae.

*The White Mouse.*—The white mouse seems to be the most satisfactory animal for initial isolation of tsutsugamushi disease from human patients. In thirteen out of fourteen cases, strains were isolated by the intraperitoneal injection of heart blood, obtained post mortem, into white mice. In the single unsuccessful experiment the sample of blood had remained in the refrigerator overnight. The patients from whom successful isolations were obtained died between the seventh and twentieth days of illness. The blood, if uncoagulated, may be injected directly or, if coagulated, may be ground and mixed with sterile saline solution. In either case, 0.3 c.c. was used for the injection.

In three out of three attempts, strains of tsutsugamushi disease were established in mice by the intraperitoneal injection of 0.3 c.c. of pericardial fluid. Pleural fluid likewise gave positive results in each of two trials, and peritoneal fluid gave a positive result in the single instance in which it was tested. Cerebrospinal fluid from the lateral ventricles gave negative results in two cases. Kouwenaar, however, isolated strains from the spinal fluid of his cases.<sup>7</sup>

Successfully infected mice appeared ill toward the end of the second week after injection and usually died before the fifteenth day. Autopsy revealed characteristic and constant changes. The peritoneal cavity was filled with cloudy mucinous fluid. The peritoneal surfaces showed marked capillary injection. The spleen was enlarged, mottled, and friable. The pleural cavities sometimes contained small amounts of cloudy fluid. The lungs were frequently congested and hemorrhagic. Giemsa-stained smears from the peritoneal exudate, spleen surface, or pleural fluid contained many large serous cells, with fewer large mononuclears and lymphocytes, and only occasional polymorphonuclear leucocytes. Typical intracellular rickettsiae were seen within the cytoplasm of the large serous cells (Fig. 15).

*The Guinea Pig.*—Guinea pigs may be infected either by intraperitoneal or intradermal inoculation and the site of infection results in slight differences in the pathologic pictures.

Infected by *intraperitoneal* inoculation with either human or wild rat strains, animals exhibit clinical evidence of illness during the second week—apathy, weakness, and dyspnea—and may die about the fifteenth day.

The gross pathologic findings are constant and characteristic. The peritoneal cavity contains upward of 10 c.c. of cloudy mucinous fluid. The peritoneal surfaces are lusterless, the capillaries injected. The spleen is enlarged and friable; its surface is lusterless and covered with delicate fibrinous exudate. The liver is enlarged and covered with a fibrinous exudate. Its external surfaces are mottled and reddish yellow and it is also friable. The kidneys and adrenals are grossly normal. There is no evidence of external scrotal swelling. The tunica vaginalis is usually without gross evidence of inflammation, although in some instances it may be slightly lusterless, its vessels injected, and occasionally punctate hemorrhage may be seen in the polar fat. The testicles appear grossly normal. The inguinal lymph nodes may be slightly enlarged and grayish pink. The pleural surfaces are smooth and glistening, and occasionally a small amount of cloudy pleural fluid may be present. The lungs show varying degrees of congestion and sometimes are hemorrhagic. The heart and brain are grossly normal.

Smears of peritoneal exudate and those from the spleen surface are rich in large serous cells, many of which contain intracellular rickettsiae within their cytoplasm.

The histopathologic findings include: (1) acute serous peritonitis with serous exudate; (2) acute splenitis with congestion and acute fibrinous perisplenitis; (3) mild acute inflammation of the tunica vaginalis with acute vasculitis and perivasculitis of the vessels of the tunica and polar fat, leucocytic infiltration, and fibrinous exudate; (4) acute fibrinous perihepatitis and acute hepatic degeneration with cloudy swelling, fatty degeneration, and focal necrosis; (5) acute renal congestion and cloudy swelling of the renal tubular epithelium; (6) varying degrees of acute pulmonary congestion, occasionally with hemorrhage; (7) mild acute focal and diffuse myocarditis, similar to lesions in the human being; (8) no typical typhus nodules in the brain, but usually marked congestion with mild perivascular round cell infiltration.

Animals inoculated *intradermally* on the anterior abdominal wall develop a primary ulcer with clinical evidence of illness during the second week—apathy, weakness, and dyspnea. In some animals death may occur about the fifteenth day.

The gross pathologic findings include a crusted, slightly red, cutaneous ulcer on the anterior abdominal wall at the site of inoculation. The peritoneal surfaces are covered with a thin layer of mucinous exudate, but little if any free fluid is present. The spleen is enlarged, friable, and deep purplish red. Its capsular surface is lusterless but without actual exudate. The liver is enlarged and friable; its capsular and cut surfaces are a mottled reddish yellow. The kidneys, pancreas, and adrenals are normal. There is no evidence of external serotal swelling. The tunica vaginalis is pale and glistening. The testicles are grossly normal.

The pleural cavities may contain small amounts of cloudy fluid, and the pleural surfaces may be covered with a sticky exudate. The lungs are usually congested with dark hemorrhagic areas throughout. The heart and brain are grossly normal. Smears of peritoneal exudate and those from the surface of the spleen show numerous large serous cells, the cytoplasm of which contain many intracellular rickettsiae.

The histologic findings include: (1) acute primary cutaneous ulcer; (2) mild acute serous peritonitis; (3) acute splenitis with congestion; (4) severe acute focal encephalitis with multiple characteristic nodules in cerebrum and pons; (5) severe acute pulmonary congestion; (6) acute hepatic degeneration with cloudy swelling, severe fatty degeneration, and focal necrosis; (7) cloudy swelling of renal tubular epithelium.

Notably absent, in contrast to the intraperitoneally infected group, is the acute fibrinous perisplenitis, the acute fibrinous perihepatitis, and the inflammation and exudation in the tunica vaginalis. This last variation indicates that the inflammatory reaction in the tunica is a result of direct extension from the peritoneal cavity. However, intradermal inoculation leads to involvement of the central nervous system and the formation of typical nodules, while intraperitoneal inoculation does not. These results are similar to those obtained in murine typhus where the serotal reaction appears to suppress the brain lesions.

*The White Rat.*—Fifteen rats injected intraperitoneally with a human strain of tsutsugamushi disease were killed and autopsied at intervals varying from the fourth to thirty-fourth day after injection. No gross or histopathologic findings could be attributed directly to the rickettsial infection in any case. There was no significant splenic enlargement as compared with control animals, and in no instance was there gross or microscopic evidence of perisplenic exudate. There was no evidence in any case of external serotal swelling, and the tunica vaginalis was uniformly pale. Microscopically there was no evidence of leucocytic infiltration in the tunica. No nodules were demonstrable in the brain. Smears from peritoneal scraping and surface of the spleen were uniformly negative.

In spite of complete lack of any illness clinically or any positive pathologic findings, the infection was shown by Kohls, Armbrust, Irons, and Philip to be persistent in the brain of each animal.<sup>15</sup>

*Bandicoots.*—Seven apparently healthy wild bandicoots injected with a human strain of tsutsugamushi disease showed no clinical evidence of illness after from twenty-one to twenty-eight days. The animals were sacrificed and

thorough autopsy study revealed no lesions attributable to rickettsial infection. The spleens were not enlarged; there was no peritoneal exudate. Smears from peritoneal scrapings and spleen surface were negative. Rickettsia could not be recovered from spleen or brain in transmission studies by Kohls, Armbrust, Irons, and Philip.<sup>15</sup> Two uninfected animals killed in the bush were found to be identical both on gross and microscopic examination with the inoculated bandicoots.

*The Wild Rat.*—Six wild rats were caught on a small island very heavily infested with rats, where many cases of tsutsugamushi disease had occurred. These rats appeared in poor health generally, as evidenced by rough thin coats, malnutrition, and sluggishness. However, there was gross or histopathologic evidence of disease due to rickettsial infection in only one instance. In this case the tunica vaginalis presented mild vasculitis and perivasculitis with moderate perivascular infiltration of mononuclear cells, and an exudate of large mononuclears, polymorphonuclear leucocytes, and fibrin over the tunica at one point. No rickettsiae could be demonstrated in tissue sections. There was no peritoneal exudate or splenic enlargement, and focal brain lesions were not found. (A strain of rickettsia was recovered by Kohls, Armbrust, Irons, and Philip<sup>15</sup> from one of several other similar rats caught at random in the same place, but no attempt was made to recover the organisms from the animals examined.)

#### COMPARISON WITH EPIDEMIC TYPHUS AND SPOTTED FEVER

Epidemic typhus and spotted fever are remarkably similar to one another histopathologically, the most striking differences being the greater tendency to thrombosis and hemorrhage in spotted fever. The presence of areas of demyelination in the brain in spotted fever<sup>14</sup> is a reflection of this greater tendency to thrombosis. In general, the tendency to thrombosis and hemorrhage appear to a smaller degree in tsutsugamushi disease than in either typhus or spotted fever.

The local cutaneous ulcer usual in tsutsugamushi disease is not seen in typhus. A similar lesion, however, occurs in *fièvre boutonneuse*, which is a strain of spotted fever.<sup>17</sup> It is noteworthy that the local lesion in the spotted fever group should be associated only with a strain of low mortality, while in the tsutsugamushi group it most often accompanies the highly virulent and fatal strains.

The tendency for the skin rash to become hemorrhagic is most pronounced in spotted fever and least pronounced in tsutsugamushi disease. These differences may be attributed to the more frequent occurrence of vascular thrombosis in the cutaneous vessels in cases of spotted fever and its relative rarity in tsutsugamushi disease; epidemic typhus occupies an intermediate position in this respect.

Involvement of serous membranes (pericardium, pleura, and peritoneum) is an outstanding feature of tsutsugamushi disease in man which is not seen in either epidemic typhus or spotted fever. In experimental animals, however, rickettsial peritonitis occurs in both typhus and spotted fever after intraperitoneal injection of rickettsiae but not after subcutaneous inoculation. In tsutsugamushi disease, the involvement of serous membranes is independent of the route of inoculation. In experimental typhus and spotted fever in the guinea pig, peritonitis is usually confined to the serosal sac, where a lower temperature obtains. In starving guinea pigs, however, generalized peritonitis, similar to that seen in tsutsugamushi disease, occurs with regularity.<sup>18</sup>

The rickettsiae of tsutsugamushi disease apparently have a greater affinity for serosal cells than those of either typhus or spotted fever.

The myocarditis in tsutsugamushi disease appears to be identical qualitatively with that seen in typhus and in spotted fever, but from our observations we believe that it tends to be rather more severe in tsutsugamushi disease. Involvement of larger vessels, including the coronaries and the aorta, is perhaps somewhat commoner and more severe than in typhus or spotted fever.

The almost constant presence of interstitial pneumonia, which, if we are correct in our interpretation, is of rickettsial origin, has not been described for epidemic typhus. Wolbach and co-workers, for example, described the lungs in regions unaffected by bacterial pneumonia as essentially normal.<sup>13</sup> It appears certain, however, that interstitial pneumonitis may occur in epidemic typhus and in spotted fever, but the lesions in these diseases apparently are not as severe as in tsutsugamushi disease.

Generalized enlargement of lymph nodes, which was present in 67 per cent of our cases, also seems more common than in typhus or spotted fever.

The changes in the brain are similar in all essential features to those seen in typhus and spotted fever. The focal lesions appear identical. The absence of areas of demyelination, such as have been described in spotted fever, is no doubt due to the infrequent occurrence of thrombotic occlusion in the cerebral vessels.

#### CLINICOPATHOLOGIC CORRELATION

All of the fifty-five patients in this series were treated in one or more evacuation or station hospitals in the forward or rear echelons of the combat zone. Overtaxed hospital services precluded the recording of any except the most essential clinical data or the performance of any except the most necessary laboratory examinations, so that the clinical information available was frequently meager. Nevertheless, an attempt has been made to correlate the clinical and pathologic features from the available data.

The pathologic findings in our series which may be regarded directly incident to the rickettsial disease are enumerated and amplified:

1. Generalized acute vasculitis and perivaseulitis occurred to some degree in all patients. On a histologic basis the vasculitis could be classified as mild with perivascular infiltration alone in 37 per cent, moderately severe with perivascular infiltration, endothelial swelling, and proliferation in 45 per cent, and severe with extensive vascular damage, hemorrhage, and occasional thrombosis in 18 per cent. To vascular damage may be attributed the punctate hemorrhages so commonly found in the various organs. More rarely hemorrhages may be sufficiently severe to cause death, as in two patients of this series. Other results of vascular damage were the small infarcts occasionally observed in the lungs, kidneys, or spleen.

2. Acute focal or diffuse myocarditis was a feature in all cases and was classified histologically as mild, without myocardial necrosis in 47 per cent. Acute serous pericarditis with or without effusion was also commonly present. Acute aortitis of mild to moderate severity was usual.

3. Acute focal encephalitis with acute serous leptomeningitis, classified histologically as moderate, with only occasional nodules or punctate hemorrhage occurred in 37 per cent; severe with numerous brain lesions in 30 per cent. Minimal changes limited to mild perivascular infiltration, hardly justifying the diagnosis of encephalitis, occurred in 33 per cent.



4. Interstitial pneumonia, believed to be of rickettsial origin, with or without acute congestion and edema, was the only pulmonary lesion in 45 per cent. A similar lesion was probably present but complicated by secondary acute bronchopneumonia in 46 per cent, while in the remaining 9 per cent such a lesion, if present, was completely obscured by severe confluent secondary bronchopneumonia. Acute serous pleuritis with effusion was present in 53 per cent.

5. Acute splenitis with marked acute congestion and enlargement of the spleen was observed in 96 per cent of the series, often accompanied by mild acute perisplenitis.

6. Acute generalized lymphadenitis was present in 65 per cent, absent in 33 per cent, and not recorded in 2 per cent. Of those cases with acute lymphadenitis, three-fourths were classified histologically as mild; one-fourth, as moderately severe with hemorrhage, focal necrosis, and moderate lymphadenopathy.

7. Acute primary cutaneous ulcer with acute regional lymphadenitis was present in 89 per cent of our cases.

8. Acute hepatic degeneration with or without enlargement had taken place in 76 per cent. Of this group, approximately three-fourths showed only mild toxic degenerative changes with cloudy swelling and fatty degeneration, while the remainder exhibited severe hepatic degeneration with acute hepatitis and focal necrosis. Moderate jaundice was present clinically in two patients and minimal in two others.

9. Acute, focal, interstitial renal lesions were present in 76 per cent of the group; in one-half of these there was also moderate focal vascular and glomerular damage and inflammatory reaction. Cloudy swelling of the renal tubular epithelium was constant.

#### CLINICAL MANIFESTATIONS IN FATAL CASES

Prodromal symptoms develop after an eight- to ten-day incubation period and consist principally of malaise, headache, chills, and fever. Between the fourth and seventh days of the febrile period a macular rash appears, first on the face, later spreading to extremities and trunk. The axillary and inguinal lymph glands are frequently painful and tender. Cough, partial deafness, and generalized body hyperesthesia are usually present. The temperature is sustained and ranges from 103 to 105° F.

Several different chains of symptoms may appear as the disease progresses:

1. Circulatory failure, evidenced by increasing pulse rate and falling blood pressure, rapid shallow respirations, cyanosis, sweating, and cold clammy skin, generally appears in the second week. This syndrome, usually diagnosed clinically as peripheral vascular collapse, closely resembles that seen in surgical shock. Less frequently circulatory embarrassment occurs which is referable to acute myocarditis.

2. Widespread central nervous system involvement is manifested by twitchings, weakness of skeletal muscle groups, delirium, convulsions, and coma.

3. Pulmonary symptoms sometime predominate. In some cases clinical and roentgenographic examination is suggestive of virus pneumonia; in others sputum examination reveals the secondary bacterial nature of the disease.

4. Rarely hemorrhage may lead to a sudden fatal issue.

5. Clinical evidence of renal insufficiency is notably absent. However, subsequent to this study, one case was observed in which edema and gross hematuria were present.

6. In addition to the symptoms which can be primarily attributed to the rickettsial disease, some patients present disturbances referable to complicating or secondary diseases. This was particularly true in the group in which the course of the disease was prolonged. Bronchopneumonia was present in approximately one-half of the group. Acute malaria was a complication in eight of our patients and suppurative parotitis in one; and nonspecific diarrhea was frequent.

Death usually occurs toward the end of the second or in the early days of the third week. In our series, 95 per cent of the deaths occurred between the ninth and twentieth day of the disease, inclusive, the average being 14.6 days.

#### CAUSES OF DEATH

Clinically, death was ascribed to circulatory failure in about one-third of the patients, to respiratory failure in about one-third, and to cerebral involvement and miscellaneous complications in the remaining third.

1. Generalized acute vasculitis was a constant finding in our cases. Woodward and Bland<sup>22</sup> recently have emphasized the importance of this lesion as a cause of death in typhus fever. Our studies indicate that their conclusions may apply equally to tsutsugamushi disease. We regard as important the concept that patients with rickettsial vasculitis may die from peripheral circulatory collapse with its attendant physiologic disturbance just as surely as though they were suffering from surgical shock. Unfortunately, the clinical study of our cases did not distinguish sharply between peripheral circulatory collapse and myocardial failure. Woodward and Bland did not encounter a single instance of congestive heart failure in their series of thirty cases, two of which terminated fatally.

2. Myocarditis was a constant microscopic finding but was considered to be severe in only about half of our patients. In patients dying early (between the ninth and twelfth days of illness), severe myocarditis was somewhat more common than in those dying later.

The myocarditis is difficult to evaluate as a cause of death. We do not believe that it is of great importance when mild. In the more severe cases of tsutsugamushi disease, however, with degenerative changes in the myocardial fibers, death may be due to myocardial failure.

Six patients in this series were treated with sulfonamides, which probably have a detrimental effect in typhus<sup>10</sup> and spotted fever.<sup>11</sup>

As pointed out by Yeomans, Snyder, Murray, Zerafonetis, and Eeke,<sup>20</sup> penicillin is the bacteriostatic agent of choice in secondary bacterial infections complicating epidemic typhus and would probably be safer in tsutsugamushi disease. Sulfonamides have been shown to cause myocarditis, rather similar to rickettsial myocarditis, in sensitive individuals.<sup>24</sup> These drugs, however, were obviously not an important factor in the myocarditis described in our patients.

3. Encephalitis was present in variable degree in practically all of our patients. Severe encephalitis and involvement of the central nervous system were definitely more common in patients dying after the fourteenth day of illness. Cerebral involvement probably is the most important cause of death in about one-third of all cases, particularly if the patient remains in stupor or coma for several days before death.

4. Renal lesions are common, but at present we cannot evaluate them as a cause of death. Correlation of the lesions with renal function is difficult, because only rarely were urinalyses or functional tests recorded. However, it may be said that of the eight patients with mild focal interstitial lesions in whom

urinalyses were reported, five showed albumin 2 to 4 plus, with hyaline and granular casts and pus cells, while of the other three patients, urinalysis on admission was negative but the test was not repeated. Of the four patients with acute glomerulitis in whom urinalysis was recorded, albumin 2 plus, with numerous granular casts and erythrocytes, was present in three, while in the fourth, urinalysis was negative on admission and not repeated.

Blood chemistry was recorded in but one patient and the blood urea nitrogen was elevated to 59 mg. per cent; the typical renal lesions were mild without severe glomerular damage. In a fatal case not of this series, nitrogen retention was marked; the inflammatory reaction was severe.

Yeomans, Snyder, Murray, Zarafonitis, and Ecker<sup>20</sup> observed that epidemic typhus frequently produced severe impairment of renal function.

5. The interstitial pneumonitis which we regard as of rickettsial origin is believed to occur almost constantly in tsutsugamushi disease. This condition probably paves the way for bacterial pneumonia, but whether it is ever alone the cause of death is problematic. However, in one case seen following this study, the clinical picture was that of pneumonia. The lung changes were very severe and entirely of the interstitial type, and it was thought that death was attributable to the primary pneumonia. It is of interest of note that clinical and roentgenologic evidence of "atypical" or "virus" pneumonia is mentioned frequently as a part of the picture of tsutsugamushi disease.

Secondary bronchopneumonia accounted for death in about 20 per cent of our cases, most frequently when death occurred between the ninth and twelfth days of illness.

6. In discussing the cause of death, mention should be made of the possible importance of toxins set free from rickettsiae and circulating in the blood. Although this factor has not been carefully studied in tsutsugamushi disease, it is probably important. It may account for the diffuse myocarditis, but probably its greatest importance lies in the effect of the toxins on the peripheral capillaries. It may play an important part, perhaps even a more important part than the rickettsial vasculitis, in bringing about peripheral circulatory collapse.

#### SUMMARY AND CONCLUSIONS

A description of the lesions of tsutsugamushi disease in man, based on the study of fifty-five fatal cases, is presented. In general, the picture is that of acute generalized endangiitis, vasculitis, and perivasculitis, the lesions resembling those seen in epidemic typhus and spotted fever but with the following differences: (1) A primary local ulcer is almost constantly present; (2) local lymphadenitis is almost constant and generalized lymphadenitis is common; (3) vascular thrombosis is less conspicuous than in typhus and much less conspicuous than in spotted fever; (4) the cutaneous eruption very rarely becomes hemorrhagic; (5) rickettsial pericarditis, pleuritis, and peritonitis are common, while these lesions are not seen in typhus or spotted fever; (6) interstitial pneumonitis, believed to be of rickettsial origin, is almost constantly present; (7) inflammatory lesions of the larger arteries, notably the aorta, are apparently more conspicuous than in typhus or spotted fever.

The strains of tsutsugamushi disease studied were readily transmitted to the white mouse and to the guinea pig by the intraperitoneal injection of blood, pericardial fluid, pleural fluid, or peritoneal fluid. A conspicuous feature of the disease in experimental animals, regardless of the route of inoculation, is the occurrence of mucinous peritonitis with many serosal cells containing rickettsiae. Such a lesion does not occur in typhus or spotted fever except in

starving animals. The pathologic picture in the guinea pig resembles that in man. A serotal reaction does not occur. Focal brain lesions were found only after subcutaneous inoculation.

Morphologically, *R. tsutsugamushi* resembles the rickettsiae of typhus and spotted fever, with minor differences which are perhaps not constant. *R. tsutsugamushi* does not distend cells as does *R. prowazeki*, but the organisms tend to form loose intracellular groups of from ten to sixty organisms.

The cause of death in tsutsugamushi disease is discussed in the light of the lesions. It is believed that peripheral circulatory collapse, emphasized as a cause of death in epidemic typhus by Woodward and Bland, is likewise of outstanding importance in tsutsugamushi disease. The myocardial and cerebral lesions are also believed to be important causes of death. Secondary bronchopneumonia was the cause of death in about 20 per cent of our patients.

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# CONGLUTINATION TEST FOR RH SENSITIZATION

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UNTIL recently, it was not clear why some individuals exhibited clinical evidence of marked sensitization to the Rh factor, yet the usual in vitro agglutination tests failed to reveal the presence in their sera of anti-Rh agglutinins. These puzzling cases have been explained, at least in part, by the discovery of the so-called Rh blocking antibodies,<sup>1</sup> which are characterized by their ability to combine specifically with Rh-positive cells but without producing a visible reaction. In fact, clinically, these blocking antibodies appear to be of greater significance than the Rh agglutinins. To detect their presence in cases where Rh agglutinins are not demonstrable, I have used the following technique: First, a drop (.05 c.c.) of a 2 per cent suspension (in terms of blood sediment) of Rh-positive red cells in saline solution and a drop of the patient's serum are mixed in a small test tube and allowed to react in a water bath at 38° C. for from thirty to sixty minutes. Then a drop of a suitable dilution of an active anti-Rh<sub>0</sub> agglutinating serum is added, and after an additional incubation period of from thirty to sixty minutes, the reactions are read. If blocking antibodies are present, no agglutination will occur, or the clumping will be markedly weakened.

In my hands<sup>1</sup> and in the hands of other investigators,<sup>2, 3</sup> the blocking test has proved a useful supplement to the usual agglutination test for Rh sensitivity. Puzzling intragroup hemolytic transfusion reactions, and instances of congenital hemolytic disease in which the maternal serum contains no demonstrable Rh agglutinins, have been clarified with the aid of the Rh blocking test. However, while the test is satisfactory as a diagnostic aid in cases of hemolytic disease, it has certain shortcomings when used as a compatibility test before blood transfusions. First, the blocking test takes twice as long as the ordinary tube agglutination tests; second, potent anti-Rh<sub>0</sub> serum is not always available. These objections have been met by Diamond and Abelson's simple slide test for Rh sensitization.<sup>4</sup> In this test, 0.2 c.c. of a concentrated (50 per cent) suspension of the test cells suspended in oxalated plasma or saline, preferably the former, is mixed with 0.1 c.c. of the patient's serum on an open slide. The test is attractive because of its simplicity, and the rapidity with which the reaction occurs is an advantage when the method is used as a compatibility test. The test is sensitive and, as Diamond and Abelson report, it gives positive results whenever either the tube agglutination or blocking tests are positive. These workers ascribed the success of their slide test to the use of heavy blood suspensions which, they stated, absorb the blocking antibodies, leaving the concealed agglutinins presumably present in the same serum free to act. Diamond and Abelson also state that the method is not suitable for determining the Rh blood types but offer no explanation for this. The slide test has the disadvantage that the mixtures dry rapidly because of the large surface, so the preparations are very perishable

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and the reactions must be read promptly. Moreover, the heavy blood suspensions and large surface on the slide favor the occurrence of troublesome rouleaux which at times are difficult to distinguish from true agglutination, while the thickness of the suspensions interferes with the microscopic reading, so that as a rule the readings have to be taken with the naked eye. The method also has the disadvantage that each blood serum must be tested separately, so that the technique is not as convenient as the tube technique for multiple testing. Accordingly, in the present paper I propose to describe a new tube test for Rh compatibility, and at the same time I shall offer an explanation for the reactions observed in Diamond and Abelson's slide test and Chown's<sup>2</sup> related capillary tube test.

The new technique of Rh testing described here differs from the standard method in the substitution of normal, compatible human plasma or serum in place of saline solution as a diluent in the tests. That is, all the blood suspensions are prepared in the usual manner and strength (2 per cent), except that serum or plasma is used as the diluent instead of saline. The serum being examined for Rh antibodies is tested undiluted or, if it is tested in dilutions as in titrations, compatible serum or plasma is used as the diluent instead of saline. For reasons which will soon become evident the name "conglutination test" is proposed for the new test, in order to distinguish it from the common agglutination test.

TABLE I. COMPARISON OF CONGLUTINATION, AGGLUTINATION, AND BLOCKING TESTS ON SERA FROM RH-NEGATIVE PATIENTS

NATURE OF TEST	TEMPERATURE OF TESTS	SERA FROM PATIENTS														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Conglutination* Agglutination*	Room	++	++	++	++	-	++	++	++	-	++	++	-	++	-	++±
		-	-	-	-	-	-	++	++	-	-	++	-	++±	-	-
Conglutination Agglutination	37.5° C.	++	++±	++	++	-	++±	++	++	-	++	++	-	++±	-	+++
		-	-	+	++	-	++	++	-	-	+	++	-	++±	-	-
Blocking†	37.5° C.	Bl.	Bl.	Bl.	0	0	Bl.	0	Bl.	0	Bl.	0	0	0	0	Bl.

\*The conglutination and agglutination tests were set up in small test tubes by mixing a drop of a 2 per cent blood suspension (type Rh<sub>a</sub>) with a drop of the serum being tested. In the agglutination tests, the blood cells were suspended in saline solution, while in the conglutination tests, the blood cells were suspended in plasma or serum.

†Bl., Blocking antibody present; 0, no blocking antibody detected.

In Table I is given a representative experiment in which the results of conglutination, agglutination, and blocking tests are compared on a series of blood sera from Rh-negative patients who were being tested for Rh sensitization. It will be seen that in every instance where agglutination was obtained in the usual tube tests, or where a positive blocking reaction occurred, strong clumping occurred in the conglutination test, while in no instance where the usual agglutination and blocking tests were both negative did clumping occur in the conglutination test.\* The conglutination reaction appears to be far less sensitive to differences in temperature than is the usual agglutination reaction. Therefore, conglutination tests may be carried out at room temperature, which is an advantage if the conglutination test is used to determine compatibility preliminary to blood transfusion.

The name "conglutination test" was selected because of the resemblance of the reactions to the so-called conglutinin (co-agglutinin) phenomenon, which has been observed particularly in tests with bovine sera.<sup>6-9</sup> When red cells

\*Subsequently, sera have been encountered which showed no Rh antibodies in the agglutination or blocking test, but in which Rh antibodies were demonstrated by the conglutination test.

(or bacteria) have been sensitized by their specific antibody and bovine serum is added to the combination, a pronounced cohesive and massy clumping of the sensitized cells occurs. Bovine serum alone has no effect unless the cells have been specifically sensitized. This phenomenon of "secondary molecular adhesion" is due to the presence in the bovine serum of a colloidal substance (possibly one or more of the serum proteins). The test described here occurs equally with fresh human serum or plasma, and with serum that has been heated at temperatures as high as 60° C. for thirty minutes, proving that the substance in question is not complement. On the other hand, even slight dilution of the serum or plasma with saline solution eliminates or weakens the reactions, probably because of the effect of the dilution on the colloidal properties of the serum. The substance apparently is adsorbed only after Rh-positive cells have been sensitized by the addition of either Rh agglutinins or Rh blocking antibodies and then brings about massive clumping of the red cells.

I believe that Diamond and Abelson's slide test and Chown's capillary tube test are both based on the same conglutinin phenomenon. Diamond and Abelson's hypothesis, that the excessive number of red cells used in the test absorbs the blocking antibody, leaving the agglutinins present in the same serum free to act, is contradicted by my observers because the reaction can also be brought about by sera containing blocking antibodies without agglutinins and in tests on diluted blood cell suspensions in serum or plasma. In their original report, Diamond and Abelson stated that the slide test could be carried out on 50 per cent cell suspensions of red cells in saline as well as plasma, but when I tried to duplicate the slide test using saline suspensions, I failed to get any convincing clumping.\* The reason for this is now obvious, because the washed blood cells diluted in saline did not contain sufficient conglutinin. With regard to Diamond and Abelson's remark concerning the unsuitability of the slide test (and this would apply also to Chown's capillary tube test) for determining the Rh blood types, this does not hold when antisera containing the agglutinins anti-Rh<sub>0</sub>, anti-Rh', anti-Rh'', and anti-Hr separately are used in the tests. On the other hand, if the anti-Rh' and anti-Rh'' reagents have been prepared from anti-Rh<sub>0</sub>' and anti-Rh<sub>0</sub>'' sera by the addition of anti-Rh<sub>0</sub> blocking serum,<sup>10, 11</sup> or if natural anti-Rh' and anti-Rh'' sera containing Rh<sub>0</sub> blocking antibodies are used, Rh typing will not be possible because the blocking antibodies themselves will bring about the clumping under the conditions which exist in the slide test and capillary tube test.

Incidentally, all the blocking sera obtained by me to date (and also by Race and Taylor<sup>2</sup>) have had specificities corresponding to anti-Rh<sub>0</sub>. Apparently, blocking sera of specificities corresponding to anti-Rh', anti-Rh'', and anti-Hr, like those described by Diamond and Abelson,<sup>3</sup> must be extremely rare.

The clinical and theoretical implications and the practical applications of the new conglutination test for Rh sensitization are manifold. First of all, it supplies the missing link to the problem of the pathogenesis of congenital hemolytic disease (erythroblastosis fetalis). Up to now, the descriptions of the pathogenesis of congenital hemolytic disease have failed to take into account the colloidal state of the infant's or fetus' own blood serum, which presumably would be quite different from that of adults. (Investigations on conglutinin in fetal and infant's sera are now in progress.) The puzzling observation that infants apparently normal at birth may suddenly exhibit clinical signs of severe hemolysis with resulting death within several hours or days may be due

\*Dr. John Elliott (personal communication) found that saline suspensions were agglutinated irregularly in the slide test. Red cells suspended in 25 per cent albumin reacted, while red cells suspended in globulin were unsuitable for the test.

to the effect of the conglutinin, which perhaps does not appear in the infant's serum until shortly before or after birth. Titration tests on maternal sera using the conglutinin technique have in a few cases revealed the presence of high-titered Rh antibodies where the usual tests showed the presence of only weak agglutinins or blocking antibodies of low titer. An important practical application is that the conglutination test, like Diamond and Abelson's slide test, makes available for use for Rh testing (determinations made with anti-Rh<sub>0</sub> antibodies only<sup>13</sup>) numerous sera which up to now have been discarded as unsuitable for use. By increasing the supply of anti-Rh serum, the conglutinin test should considerably increase the clinical usefulness of the Rh diagnostic tests; for example, for prenatal testing of pregnant women.

While the technique described deviates from that customarily used for Rh agglutination, actually it is simpler in certain respects. First, the reactions appear to be almost as strong at room temperature as at body temperature so that use of a water bath or incubator is unnecessary. Second, it is unnecessary to wash the cells; in fact, washing the cells is to be avoided. While it is desirable to obtain the blood to be tested by venipuncture, this is not essential if this is not feasible, because the red cells can be suspended in group AB serum (or plasma) stored in the refrigerator or any serum of a compatible group. Blocking serum can be used as an anti-Rh<sub>0</sub> agglutinating reagent like ordinary anti-Rh<sub>0</sub> serum, if the isoagglutinins they contain are neutralized by the addition of dried A and B substances,<sup>14</sup> and the sera are diluted to the desired titer with inactivated group AB serum instead of saline. Of course, the blood cells being tested must be suspended in serum instead of saline.

The observations reported may serve to reconcile the opposing views concerning the specific or nonspecific nature of the second stage of the agglutination and precipitation reactions.<sup>15</sup> With Herman, I have observed<sup>16</sup> that in mixed agglutination systems where diluted sera are used, the agglutination is specific so that each clump contains cells of the same kind only. In tests with undiluted sera, on the other hand, mixed clumps were obtained. Probably, in the latter instance we were dealing with the conglutinin phenomenon rather than with a typical agglutination reaction. It seems that in typical agglutination reactions the second stage as well as the first stage is specific, while in conglutination reactions the second stage is nonspecific. Similar effects probably hold in the case of precipitation reactions,<sup>17</sup> except that in the precipitation tests undiluted serum is used more often than in agglutination tests.

Experiments are in progress to apply the conglutination reaction to other antigen-antibody systems. For example, it may prove possible to improve the speed and intensity of the reactions of anti-A and anti-B grouping sera with the aid of this phenomenon. In fact, it seems likely that the increased avidity which is exhibited by the antisera prepared by globulin fractionation may be explained on this basis. As another example, the test may prove useful in explaining the pathogenesis of the so-called acquired hemolytic anemias.<sup>18</sup> Under certain conditions, a sudden breakdown of blood cells may occur; for example, due to infection with viruses having an affinity for erythrocytes, or the action of hemolytic poisons (sulfanilamide, fava bean, etc.), during the crisis of familial hemolytic icterus, from infectious agents (malarial parasites, streptococcus hemolyticus infections, etc.), trauma (hemorrhage into tissues with formation of large hematomas), etc. As a rule, after recovery from the acute insult no sequelae result, but in rare susceptible individuals, one could conceive that the elimination of the destroyed red cells may result in the production of auto-antibodies. These autohemolysins could destroy more of the individual's red



cells, and a vicious cycle could result with the picture of a protracted hemolytic anemia sometimes continuing long after the initial noxious agent has ceased acting. One difficulty with this theory is that attempts to demonstrate auto-antibodies in the patient's serum have up to now yielded equivocal results in the great majority of cases. Thus, when autoagglutinins were found, these mostly were active only at low temperatures and so did not differ from the cold autoagglutinins occurring normally in the sera of most individuals, except for their titer. Recently, in a case of acquired hemolytic anemia, by using the conglutination technique, I succeeded in demonstrating autoagglutination *in vitro* at body temperature as well as in the refrigerator. If in future cases of acquired hemolytic anemia the conglutination test proves equally successful, this may help solve the problem of the pathogenesis of these obscure anemias.

## COMMENT

It may be of interest to review how the test-tube conglutination test for Rh sensitization described here was developed. When the report of Diamond and Abelson appeared, describing their important slide test for Rh sensitization, naturally an attempt was made to reproduce their results in our laboratory. When 50 per cent blood suspensions in saline solution were used, one of the alternative methods mentioned in their article, the results were unconvincing. A visit was then paid to Dr. Diamond's laboratory at his invitation, where he gave a most impressive demonstration of the test. When again, upon returning to our laboratory, despite Dr. Diamond's demonstration and explanation of the slide test, we were still unable to duplicate his results, it was decided to investigate the mechanism of the reaction in an attempt to explain the discrepancy. It was then found that it was not the strength of the blood suspension but the avoidance of the introduction of saline solution into the mixture that spelled the difference between success and failure in the test.

For practical purposes, as in compatibility tests before blood transfusions, or wherever a rapid diagnosis is essential, the slide test of Diamond and Abelson will be the method of choice. The conglutination test herein described may prove most useful for more precise work, particularly in more exacting theoretical investigations.

While this paper was in press, an investigation was started to determine the results of slide tests and tube conglutination tests on the serum of infants with hemolytic disease. With the aid of these techniques, Rh antibodies have thus far been demonstrated without difficulty in three successive cases. Significantly, the blood cells of these infants were only weakly agglutinated or failed to agglutinate with standard anti-Rh<sub>0</sub> serum in agglutination tests carried out by the customary tube technique, presumably due to adsorption onto the surface of the infant's cells of Rh<sub>0</sub> blocking antibodies derived from the mother through the placenta. In support of this idea was the observation that in the slide test and/or tube conglutination test, autoagglutination of the infant's cells by its own serum was readily demonstrated. This also explains the occasional paradoxical reports submitted by laboratories unaware of this phenomenon that the infant as well as the mother is Rh negative (or belongs to type Rh' or Rh'') even though the infant has hemolytic disease. Investigations are being conducted to determine how long the Rh antibodies persist in the infant's circulation. In one infant, treated by two transfusions of a total of 200 c.c. of Rh-negative blood, Rh antibodies were still demonstrable two weeks after birth in tests carried out with dilutions of the infant's serum as high as 1:5.

In the meantime, our attention has also been called to the important monograph of K. O. Pedersen (Ultracentrifugal Studies on Serum and Serum Fractions, Uppsala, Sweden, 1945), in which are described observations of significance in relation to the conglutination test. Pedersen discusses in detail a so-called X protein, which makes up from 20 to 50 per cent of the total serum proteins and is a protein of very high molecular weight consisting of a complex of albumin, globulin, and phospholipid. X protein dissociates readily

upon slight dilution with saline solution, like the colloidal substance responsible for the conglutination reaction, and observations on at least one individual suggest that it may be increased as a result of immunization. Moreover, in cow's fetus and newborn calves and foals, Pedersen has found a globulin of low molecular weight, named fetuin by him, which may be a precursor of one of the constituents of X protein. Fetuin is present only in small amounts in human umbilical cord blood, which is in line with the suggestion made in this paper that there may be a change in the colloidal properties of human serum toward the end of pregnancy or at about the time of birth.

## SUMMARY

A new tube test (conglutination test) for Rh sensitization has been described. The technique entails the use of red cells suspended in compatible human plasma (or serum) instead of saline solution, while the serum being examined for its agglutinin content is tested either undiluted or diluted with plasma or serum. Dilution of the mixture with saline solution or any other crystalline solution is strictly avoided. Positive reactions are obtained in all cases where the serum being tested contains Rh agglutinins detectable for the usual tube technique and/or Rh blocking antibodies, so the results are similar to those obtained by the slide technique of Diamond and Abelson. The reaction seems to depend upon a thermostable colloidal substance in the serum (or plasma) which brings about massive agglutination of Rh-positive cells but only after they have been sensitized by their specific antibody.

The observations described supply one of the missing links in the theories of the pathogenesis of congenital hemolytic disease and intragroup hemolytic transfusion reactions. Moreover, like Diamond and Abelson's slide test, they help increase the supply of Rh testing serum available for clinical use by rendering useful sera that are of no value in tests performed by the customary techniques.

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## THE DETECTION OF RH SENSITIZATION: EVALUATION OF TESTS FOR RH ANTIBODIES

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THE detection of Rh sensitization is a problem with which those who deal with blood transfusion or with obstetric patients are frequently faced. As we<sup>1</sup> have previously pointed out, the laboratory recognition of such sensitization may avert or resolve hemolytic transfusion reactions. All too frequently, patients are exposed to the discomforts and hazards of incompatible transfusion, or denied further transfusion, because a previous reaction has not been satisfactorily explained. A substantial number of these patients can be treated successfully if the clinician has recourse to adequate means of detecting the source of incompatibility. Furthermore, the employment of a reliable method of detecting Rh antibodies is an important aid in the management of pregnancies of Rh-negative women whose husbands are Rh positive. Many couples who have learned of the importance of the Rh factor, but who have acquired inadequate information or misconceptions concerning it, come to the physician with questions and with apprehensions with which he can deal honestly and capably only if he has at his disposal satisfactory methods for Rh typing and for determination of Rh antibodies. Trustworthy reports of the absence of antibodies are reassuring both to the physician and to the patient. Unfortunately, the idea has become prevalent that most of the complications of pregnancy (and particularly miscarriages and abortions) in Rh-negative women are due to Rh incompatibility. This misapprehension can best be corrected by the provision of accurate and convincing laboratory data. Moreover, the demonstration that antibodies are present serves as a guide for prognosis and permits mobilization of the safeguards now available for the mother and the erythroblastotic infant, thereby converting these deliveries from unpredictable emergencies into medical problems over which a certain amount of control can be exercised.

In the past, the laboratory confirmation of Rh sensitization has usually been dependent upon a single test which has proved inadequate in as many as from 40 to 50 per cent of instances. Laboratory means for detection of Rh antibodies have, therefore, gained a poor reputation. However, not a single, but several, methods of detection are now available, and it seems worth while to assess their value as laboratory aids to clinical medicine. These tests are conveniently discussed herewith.

### THE BIOLOGIC TEST

The biologic test has been proposed and used by Wiener and co-workers.<sup>2, 3</sup> It involves the injection of a quantity (50 c.c.) of Rh-positive cells into the Rh-negative patient suspected of possessing abnormal antibodies, or the in-

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jection of this amount of blood from a proposed donor into a patient who has had a previous transfusion reaction. After an hour, the patient's plasma and serum are compared with plasma and serum drawn at the beginning of the test. If there is a change in the depth of the color of the plasma and serum (that is, an increase either in bilirubin or in free hemoglobin), the blood given is assumed to be incompatible. If there is no alteration, and an emergency exists, a full transfusion is given. If time permits, however, the test is repeated with a second 50 c.c. Care must be taken that factors tending to hemolyze the red cells are eliminated or avoided, and it is for this reason that specimens of both serum and plasma are withdrawn.

The test has obvious clinical disadvantages. Besides necessitating several venipunctures over a considerable period of time, it presents, first, the immediate danger of transfusion reaction. Highly sensitized patients (whose antibodies have in the past often been difficult to detect *in vitro*) may have a moderate reaction to as little as 5 or 10 c.c. of incompatible blood, and the reaction to as much as 50 or 100 c.c. may be one of serious proportions. In one case which we observed, chills, fever, and abdominal pain followed the intravenous injection of only 5 c.c. of packed red cells into a patient who had previously been delivered of a baby with erythroblastosis fetalis. Second, the injected red cells are potentially antigenic. There is no doubt that the infusion of 50 or 100 c.c. of Rh-positive blood into an Rh-negative person (and especially a woman in the childbearing age) may be a factor in initiating or restimulating antibody production. The use of the biologic test is, therefore, no longer justified except in the rare case where *in vitro* methods may yield equivocal results. As Weiner<sup>3</sup> has pointed out, this test was never meant to supplant, but rather to supplement, the *in vitro* detection of sensitization and was intended only for emergency situations when laboratory assistance is not readily available. It is especially no longer necessary since the open slide test and its test-tube modification (described later) are now available for dependable determination of Rh antibodies.

#### THE MODIFIED COMPATIBILITY, OR TUBE-INCUBATION, TEST

Levine<sup>4</sup> found that Rh agglutinins studied by him reacted more intensely at 37° C. than at 20° C. and suggested that incubation was essential for their detection.<sup>5</sup> He recommended that in performing compatibility tests in cases where isoimmunization might be a factor, the patient's serum and the prospective donor's cells be incubated for from fifteen to thirty minutes at 37° C. The method has been modified for detection of Rh antibodies as follows:

Two drops of a 2 per cent suspension of Group O, Rh-positive red cells in normal saline (roughly one full drop of blood to about 1 c.c. of saline) are placed in a small test tube. Two drops of the unknown serum are added. After shaking, the tube is placed in a 37° C. water bath for one hour, then gently agitated and centrifuged at 500 revolutions for one minute. The character of the cell button is noted (a smooth outline is characteristic of the negative test). The tube is then gently tilted a half dozen times, to loosen some of the sediment from the bottom of the tube, and observed. Gross clumping can be seen with the naked eye or with the aid of a hand lens. If no gross clumping is observed, a drop of the suspension is examined microscopically for confirmation of absence of agglutination. The result is usually controlled by testing also with an Rh-negative cell, and the specificity of the serum is determined by testing with several different Rh-positive cells (some

belonging to the phenotype Rh<sub>0</sub>' and some of the phenotype Rh<sub>0</sub>''). By experienced technicians, sensitization may thus be detected in a substantial number of the cases. With inexperienced workers, this test is much less satisfactory, since in many cases the agglutinates are so fine as not to be apparent to the uncritical eye and so fragile as to be easily broken up by careless or vigorous handling. In an analysis of 500 cases of Rh sensitization (proved by hemolytic transfusion reaction or the delivery of an erythroblastotic infant), 265 sera (53 per cent) produced moderate or strong agglutination with the majority of Rh-positive cells. In addition, 57 sera (11.2 per cent) produced very weak agglutination, and 22 (4.4 per cent) produced agglutination only with cells of specificity Rh<sub>0</sub>'.

This test, despite its limitations, has certain important advantages. If the physician has had the opportunity to follow the patient from the onset of sensitization, and particularly if quantitative estimations of the amount of antibody present in the blood stream are regularly made (that is, titration by the serial dilution method), it provides a useful guide to prognosis. Generally speaking, if only agglutinating antibodies are found by this method, and if these have begun to increase in strength only in the latter months of pregnancy, the fetus has a fair chance of survival. In these cases the mother may be spared further sensitization, and the infant further damage, by a reasonably early induction of labor. On the other hand, a single test showing the presence of agglutinins does not lend itself so easily to clinical interpretation, since a low titer of agglutinating antibodies (as determined by this method) may denote either early sensitization or profound late sensitization, depending upon the presence of inhibitor substances or "blocking" antibodies (described later and elsewhere). For this reason, sera of pregnant, Rh-negative patients should be tested at regular intervals.

#### THE "BLOCKING" TEST

The "blocking" test has recently been proposed by Wiener.<sup>6</sup> It is based upon a discovery made independently by Race<sup>7</sup> and by Wiener that many persons sensitized to the Rh factor develop antibodies which are capable of combining with the Rh-positive erythrocyte without a visible reaction, and which are, in fact, capable of preventing the usual visible reaction with potent, standard (Rh<sub>0</sub>) sera. As originally described by Wiener, this test was performed by addition of the unknown serum to a 2 per cent suspension of Rh-positive cells, followed by incubation in the water bath at 38° C. for from thirty to sixty minutes. The tubes were then centrifuged and, in some cases, the supernatant fluid withdrawn. The results of the test were not significantly altered by this latter procedure. A drop of potent anti-Rh<sub>0</sub> serum was thereupon added and the mixture incubated for another thirty to sixty minutes, following which, after centrifugation, the tubes were observed for inhibition of the expected agglutination. We have found it possible to shorten the procedure and to perform the test in conjunction with the tube-incubation method for agglutinins. At the same time the latter test is set up, two additional suspensions of Rh-positive cells are prepared. The cells are so selected that one belongs to the phenotype Rh<sub>0</sub>' and the other to the phenotype Rh<sub>0</sub>''. The unknown serum (2 drops) and the potent agglutinating serum (1 drop of a dilution of titer 4 to 8 or whatever final dilution just yields grossly visible agglutination) are added at the same time. A parallel control is employed, using saline instead of the unknown serum specimen. The tubes are then incubated and centrifuged in the same manner as described, and search for

agglutination is carried out. If agglutination occurs in the control tube but is absent or inhibited in the tube containing the unknown serum, blocking antibodies may be assumed to be present. There are several points to which particular attention must be paid in the performance of this test:

1. The Rh-positive cells used should belong to the phenotypes Rh<sub>1</sub> (or Rh<sub>0</sub>' ) and Rh<sub>2</sub> (or Rh<sub>0</sub>'').

2. The specificity of the agglutinating serum should be carefully determined, because by far the greatest number of blocking antibodies are effective only against Rh<sub>0</sub> sera. An Rh<sub>0</sub>' or Rh<sub>0</sub>'' serum should not be used; otherwise the Rh' and Rh'' agglutinins will interfere with the Rh<sub>1</sub> (or Rh<sub>0</sub>') and Rh<sub>2</sub> (or Rh<sub>0</sub>'') cells, respectively.

3. The test should not be set up without a control; otherwise partial inhibition of the expected agglutination may not be appreciated.

In our analysis (see Table I), 120 sera (24 per cent) produced strong inhibition, 57 (11.4 per cent) produced weak inhibition, and 22 (4.4 per cent) produced inhibition with only the Rh<sub>0</sub>'' cell.

This method, like the tube-incubation test for agglutinins, is a valuable index of Rh sensitization. It is now evident that the presence of the blocking antibody does not mean that the individual has undergone desensitization; on the contrary, it denotes a high degree of immunization. The recipient is then likely to have extremely severe reactions from infusion of incompatible blood, and the infants of women whose sera contain these antibodies are as severely damaged, if not more profoundly affected, than those of women whose sera produce strong agglutination by the usual tube test. Moreover, since the presence of this substance denotes a high degree of antibody production and certain disaster to the Rh-positive fetus, repeated quantitative estimations of amounts present in the blood stream are probably more of academic than of clinical importance. This is in contrast to tests for ordinary agglutinins, the quantitative serial estimations of which have proved of considerable value in following maternity cases.

TABLE I. ANALYSIS OF 500 RH ANTISERA

	NUMBER	PERCENTAGE
Sera producing only strong agglutination (in test tube with 2% suspension of red blood cells in saline)	64	12.8
Sera producing only weak agglutination (in test tube with 2% suspension of red blood cells in saline)	201	40.2
Sera producing only strong inhibition (in test tube with 2% suspension of red blood cells in saline)	120	24.0
Sera producing strong agglutination and strong inhibition*	22	4.4
Sera producing weak agglutination with weak inhibition	57	11.4
Sera reacting only on the open slide	36	7.2

\*These are sera of the so-called "70 per cent" or anti-Rh' variety. They contain Rh' agglutinins demonstrable in the test tube and an antibody which inhibits Rh<sub>0</sub> agglutination. The type of reaction in the test tube, therefore, depends upon the specificity of the red cell against which it is tested.

#### THE COMBINED TUBE-INCUBATION TESTS FOR AGGLUTINATING AND BLOCKING ANTIBODIES

When the two tests described above are performed in parallel, sensitization can be detected, by careful workers, in most of the cases. The analysis to which we have already referred (Table I) shows that the combined methods indicated sensitization in 464 instances (92.8 per cent). The tests have the disadvantage of being somewhat laborious. They require a good deal of equipment, material, and particularly experience. However, the usefulness of the information they provide offsets the disadvantages.

## THE CAPILLARY TUBE TEST

Chown<sup>8</sup> has described an economical and ingenious method for Rh typing which is applicable to the detection of Rh agglutinins. Capillary tubes of 0.4 mm. bore and approximately 8 cm. in length are sterilized in test tubes. One end of the capillary is dipped into the serum to be tested, and a column approximately 2 cm. in height is permitted to run in. The capillary is next dipped into fresh, Group O, oxalated or citrated blood, making sure there is no air bubble between serum and blood. The tube is then inverted to allow the mixture to run to the opposite end of the tube. The blood-containing end of the capillary is inserted into a plasticine rack at an angle of 45 degrees to the horizontal. After incubation at 37.5° C., the test is read against a white background. With a positive test, a beaded layer is formed along the lower side of the capillary. A negative test produces a thin, smooth line. This method seems to depend on factors similar to those underlying the open slide test (to be described). It is reliable when properly employed, but it may be difficult for the average laboratory worker to read. It also possesses no differential value. Its greatest advantage lies in the fact that it requires a minimum of time, equipment, and material, especially serum. Also, like our slide test, it should detect evidence of Rh sensitization by agglutination, even in the presence of "blocking" antibodies.

## THE OPEN SLIDE TEST\*

The open slide test was recently described by Diamond and Abelson.<sup>1</sup> Fresh, whole Group O, Rh-positive blood of normal hematocrit is mixed with about an equal amount of serum (approximately 0.2 c.c.) on the open slide, which is moderately warmed over a 25-watt bulb. The slide is gently tilted from time to time. After a short interval (varying from a few seconds to three minutes), if the test is positive, readily discernible agglutinates appear. A parallel control of Rh-negative blood is highly desirable, particularly in doubtful cases.

Errors in interpretation are chiefly due to nonspecific agglutination or rouleaux formation, which yields a slightly granular appearance to the preparation, but experience helps differentiate this readily from true agglutination, which is much more marked. A drop of saline added to the questionable result with restirring and agitation shows a clear difference between true agglutination and rouleaux formation due to drying.

In the original description<sup>1</sup> it was suggested that once-washed red cells resuspended in saline were satisfactory for performing this test. Further extensive experience has shown that thoroughly washed red cells (two or more times) fail to react unless they are resuspended in plasma or albumin. Only the cells incompletely washed free of their original medium will permit agglutination on the slide. For this reason it is necessary to use oxalated or citrated blood unwashed, or, if washed, resuspended in plasma, serum, or, as recently found most satisfactory, albumin.

At first it appeared that this test was successful because of the fact that sufficient antigen in the form of Rh-positive red cells was provided to absorb "block-

\*Dr. A. S. Weiner, to whom this test was demonstrated in April, has confirmed in his own laboratory that even the most highly "blocked" sera produce agglutination by this technique. In his discussion of this test at the conference on Blood Grouping, held at the New York Academy of Sciences, May 18, 1945, he used the term "conglutination," rather than agglutination, for this phenomenon to distinguish it from clumping which occurs with a saline suspension of red cells. Since the "conglutination" reaction between antibody (in serum) and antigen (in the red cells) is in no way grossly different from the agglutination which occurs in the test tube, the clumping of Rh-positive cells by the anti-Rh antibody (either of the  $\alpha$  or  $\beta$  type) taking place *in vivo* has always been designated as agglutination, the question might well be raised as to what term should be used in the future. Certainly these observations may make it necessary to change our concept of the agglutination reactions which occur within the body.

ing antibodies" present in the serum, thus permitting the action of "masked" agglutination. This theoretic explanation no longer seems valid.

A discussion of the principles underlying the slide test is presented in another paper.

The following factors appear to accelerate the slide reaction:

The presence of unwashed red cells, or washed red cells resuspended in serum, plasma, or albumin; the presence of an excess of antigen with gentle agitation and mild warming, both of which make clumping more rapid and more easily detectable; the presence of a glass surface. Landsteiner,<sup>9</sup> in discussing nonspecific factors in the second stage of antigen-antibody reactions, wrote, "The fact that agglutinated cells may stick to the walls of test tubes, and that sensitized cells tend to adhere to leucocytes or to platelets, may possibly be caused, in keeping with the lattice theory, by antibody adsorbed to the glass surface, and perhaps on cells." He referred to Mudd's statement<sup>10</sup> that he "has observed in many years of experience with the interfacial technique that specifically sensitized bacterial and other cells are sticky, not only for each other, but for glass slide and coverslips as well . . ."

A modification of the principles employed in this open slide test method has been adapted by us to test tube use. This consists of the suspension of red cells washed, or unwashed, in plasma, serum, or, as a best medium, human or bovine albumin.\* Under these circumstances a concentration of red cells varying from 2 to 5 per cent in such a blood protein diluent can be used. Mixture of one or two drops of this red cell suspension with an equivalent amount of the "blocked" serum will yield, after short incubation and centrifugation, visible agglutination. The unknown serum can even be diluted with plasma, serum, or, preferably, albumin and a titration of its strength carried out by this method. In routine use it has been found that the slide test can be carried out more rapidly and yields equally accurate results, so that it is most suitable for use in a large laboratory.

When the precautions mentioned are heeded, the slide test is reliable, simple, and speedy in result. In the Blood Grouping Laboratory it has failed to confirm Rh sensitization only three times in a series of over 1,200 tests; of the 500 cases referred to previously, only one of proved sensitization was missed by this means. An analysis is presented in Table I and a comparison of the three methods in use in our laboratory for detection of Rh sensitization is found in Table II.

TABLE II. COMPARISON OF THREE IN VITRO TESTS FOR DETECTION OF ANTIBODIES IN 500 CASES OF RH SENSITIZATION

	NUMBER	PERCENTAGE
Cases detectable by the tube-incubation test for agglutinins	344	68.8
Cases detectable by the blocking test (often with weak agglutinins)	199	39.9
Cases detectable by the combined agglutination and blocking tests	464	92.8
Cases detectable by the open slide test	499	99.8

From Table II it will be seen that when used alone, neither of the earlier tube tests is as satisfactory as the open slide test for detection of Rh sensitization. The latter test does not, however, indicate specificity of sera, according to Wiener's classification. By its use, no distinction can be made between early and late sensitization, blocking and agglutinating antibodies. Its chief value, therefore, is as a screen test in following obstetric patients, or as a con-

\*Details of the study of Rh agglutination using albumin as a diluent are presented in a paper to be published shortly.



firmatory test when tube demonstrations using saline cell suspensions are equivocal. In resolving hemolytic transfusion reactions, we have found it to be of value in detecting incompatibilities other than those due to the Rh factor; in particular, incompatibility between A<sub>2</sub> and O or A<sub>1</sub> bloods. For this reason, and because it is in general more sensitive and more reliable than other methods, its use is particularly indicated in dealing with problems involving transfusions and transfusion reaction. More important, it has led to a new understanding of the nature of the "blocking antibodies" so often found in persons sensitized to the Rh blood factor and has suggested new explanations for some of the hitherto puzzling features of many cases of erythroblastosis fetalis. In addition, the demonstration of agglutination of red cells suspended in plasma, serum, or albumin by an antibody which fails to yield visible agglutination of red cells suspended in saline, the time-honored diluent for agglutination tests, has offered a new technique for study. It suggests the need for re-evaluating previously obscure hemolytic reactions. It is a particularly logical technique since within the body, red cells are suspended in blood protein diluents, not in saline alone, and agglutination in vivo may involve not only the red cells and the antibody, but also the blood fluids.

#### SUMMARY

The various tests available for detection of Rh sensitization are (1) the biologic test, (2) the modified compatibility or tube-agglutination test, (3) the "blocking" test, (4) the capillary tube test, and (5) the open slide test.

The biologic test possesses very limited clinical usefulness and should not be used to supplant, but rather to supplement, the in vitro methods. Of the latter, the open slide test, when used alone, is by far the simplest and most reliable. It is particularly valuable as a screening test in following obstetric patients and as a confirmatory test for compatibility of blood donor and recipient. For a more refined diagnosis, the tube-agglutination and blocking tests used in conjunction with one another possess a high degree of reliability and yield valuable information concerning the stage of immunization to which the sensitized patient has progressed.

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## STUDIES IN HODGKIN'S SYNDROME

### III. THE RELATIONSHIP OF TUBERCLE BACILLI TO HODGKIN'S SYNDROME

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THE possible etiologic relation of the human tubercle bacillus to Hodgkin's syndrome was first suggested by Sternberg<sup>1</sup> in 1898. Twenty-six years later, the recovery of the avian tubercle bacillus by L'Esperance<sup>2</sup> reopened the question of a possible tuberculous etiology in this disease. The present study is an attempted re-evaluation of the etiologic role of human, bovine, and avian tubercle bacilli in Hodgkin's syndrome and is one phase of a broad, general resurvey of the Hodgkin's problem now in progress in this laboratory.<sup>3-5</sup>

In addition to the reports of Sternberg<sup>1</sup> and L'Esperance,<sup>2</sup> a large number of papers have appeared both in support and in refutation of their conclusions. The voluminous literature has been reviewed by Wallhauser (human and bovine tubercle bacillus),<sup>6</sup> Feldman,<sup>7</sup> Branch,<sup>8</sup> and Steiner (avian tubercle bacillus)<sup>9</sup>; see also our recent discussion of the incidence and geographic distribution of cases of Hodgkin's disease reported in the United States of America.<sup>3</sup>

#### MATERIAL AND METHODS

Six varieties of media were chosen for the attempted isolation of acid-fast organisms:

1. Corper's medium,<sup>10</sup> containing 5 per cent glycerine, malachite green, and Sorenson's phosphate mixture (pH 7.2) (buffer).<sup>11</sup>
2. Corper's medium without buffer and with crystal violet.
3. Egg yolk,† Sorenson's phosphate mixture pH 7.2 (buffer), 5 per cent glycerine, 1 per cent extract of killed human tubercle bacilli and malachite green.
4. Long and Seibert's synthetic medium<sup>12</sup> with 10 per cent serum and malachite green.
5. Petragani's medium<sup>13</sup> with malachite green.
6. Herrold's medium<sup>14</sup> with malachite green.

It was found that the growth of the two human strains, II 37 and 100.1†, was most luxuriant on media 1 and 5.

Avian strains 7992, 3746, 3741, and 3296-76§ grew most luxuriantly on medium 3, a modified Corper's medium containing an extract of tubercle bacilli. With these results in mind, media 1, 3, and 5 were chosen for the projected growth of acid-fast organisms from Hodgkin's tissue. Thirty lymph nodes and three spleens were obtained from thirty-three patients with histologically proved

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†Modification of Corper's medium containing extract of killed human tubercle bacilli.

‡H 37, *Mycobacterium tuberculosis* human. 100.1, *Mycobacterium tuberculosis* human, obtained from the curator of cultures, Department of Bacteriology, Ohio State University.

§7992, *Mycobacterium tuberculosis* avium, obtained from National Type Culture Institute, Georgetown University, Washington, D. C. 3746, 3741, and 3296-76, *Mycobacterium tuberculosis* avium, obtained from Dr. W. H. Feldman, The Mayo Foundation, Rochester, Minn.

Hodgkin's disease for use in the direct inoculation of media. Direct smears stained to demonstrate the presence of acid-fast material were made in each case before the seeding of media. All inoculated tubes and plates were tested at regular intervals for a period of three months whether bacterial colonies appeared to be present or not.

Sixty-six guinea pigs were inoculated subcutaneously in the region of the groin with a thick emulsion of Hodgkin's tissue. Guinea pig controls were similarly inoculated with lymphatic tissue emulsions obtained from fifteen patients with diseases of diverse etiology. Thirty Leghorn chickens were inoculated intravenously with Hodgkin's tissue emulsion, and ten control Leghorn chickens were inoculated with tissue emulsions from other disease sources. Eight rabbits, four control and four experimental, were inoculated in a similar manner. All animals were skin tested with avian O.T. and human P.P.D. tuberculin before inoculation and at intervals during the experimental period. The minimum interval between successive skin testing with avian O.T. was six weeks.

Another group of guinea pigs (twenty in number) was sensitized with 1 to 3 mg. of killed human tubercle bacilli (dry weight) according to the method of L'Esperance<sup>15</sup> eight to twelve days previous to inoculation with Hodgkin's tissue emulsion. Ten additional pigs received only killed tubercle bacilli and were used as controls.

All experimental animals were carefully autopsied either at death or at an elected period eight months following inoculation. An attempt was made in every animal to inject the largest amount of tissue possible in order to obtain the largest obtainable inoculum of acid-fast organisms if present. Animals of both sexes were used in each experimental group. Fixed tissue sections were made from liver, spleen, lung, lymph nodes, and other tissues as indicated. All lesions of a suspicious nature were cultured for acid-fast organisms.

#### RESULTS AND DISCUSSION

No acid-fast bacilli were isolated either on direct inoculation of artificial media with human tissue inoculum or from autopsy material in the case of the experimental animals, and no macroscopic granulomatous lesions were observed in any of the experimental animals except under the following circumstances:

Four cases in the patient control series were diagnosed histologically as tuberculosis, and in all four cases human tubercle bacilli were obtained from inoculated media and/or from inoculated guinea pigs.

In one of the guinea pigs inoculated with tissue obtained from a patient with Hodgkin's sarcoma there was found at autopsy, eight months after inoculation, the typical macroscopic appearance of advanced Hodgkin's disease of the human liver in miniature. Microscopically, however, the lesion appeared to be fibrous rather than granulomatous and no reticulum cell hyperplasia was present.

Results of skin tests in guinea pigs, chickens, and rabbits were negative except in those cases mentioned in which acid-fast organisms were isolated.

From the results obtained during the present course of study it is not possible to say that the avian, bovine, or human tubercle bacillus, sought under the conditions specified, has an etiologic role in Hodgkin's disease. It is possible, of course, that there may be acid-fast organisms which are difficult or impossible to cultivate on media thus far described; and it is also considered possible that the presence of tuberculo-toxins may contribute in some manner, in perhaps specially sensitized tissues, to the development of the granulomatous lesions observed in Hodgkin's disease. Although it is an established fact that tubercle bacilli may be cultivated at autopsy, in from 15 to 20 per cent of patients with

Hodgkin's disease, and that the immunologic data of Stewart and Doan<sup>16</sup> suggest the not infrequent presence of a concomitant tuberculous process during life, it cannot be concluded that this evidence in any sense establishes a causal relationship between the two diseases. There are many other possible explanations for the coincident presence of the two processes in one individual during life and at post-mortem.

In most cases the tissues studied in the present series were removed because of the necessity for diagnostic biopsy, and the results, therefore, cannot be compared with similar studies in which the material was obtained from patients with advanced Hodgkin's disease or at post-mortem.

#### SUMMARY AND CONCLUSIONS

A series of chickens, guinea pigs, and rabbits have been inoculated with fresh macerated lymph node and splenic tissue emulsions obtained surgically from patients with Hodgkin's syndrome and other diseases of widely differing etiologies. In each case carefully chosen media were inoculated with tissue emulsion. In no instance were tubercle bacilli found except in the case of histologically, bacteriologically, and clinically proved tuberculosis.

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## STUDIES IN HODGKIN'S SYNDROME

### IV. THE THERAPEUTIC USE OF RADIOACTIVE PHOSPHORUS

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THE use of radioactive phosphorus in the treatment of Hodgkin's syndrome has been referred to briefly by Low-Beer and co-workers,<sup>1</sup> Hemplemann and associates,<sup>2</sup> and Fitz-Hugh and Hodes<sup>3</sup> in reports devoted to its use in leucemia and so-called allied diseases. The largest series<sup>1</sup> reported (nineteen cases) is representative of other published information on this subject, and the authors concluded that temporary favorable responses occurred only in patients who had "slight manifestations" and that the maximum period of induced remission was one year. Published data on the use of radioactive phosphorus and x-ray therapy in the same patients either concomitantly or successively are so meager that no deductions are justified.

#### MATERIAL AND METHODS

Radioactive phosphorus used in this study was prepared in the Ohio State University cyclotron under the supervision of Dr. M. L. Pool. Red phosphorus was bombarded with a ten million volt deutron beam of approximately 100 micro-amperes. The original material was oxidized with nitric acid and the dibasic salt of phosphorus was formed with the addition of sodium hydroxide. The solution was administered in an isotonic form, and its initial activity was approximately 200 microcuries per cubic centimeter. Radioactivity was measured with the Lauritsen electroscope immediately following bombardment.

Eleven patients with Hodgkin's disease, proved by histologic examination of tissue obtained at biopsy, were chosen for study; four were males, and seven were females. The age of each individual and the duration of symptoms before treatment with radioactive phosphorus are indicated in Table I.

A careful evaluation of the clinical status and hematologic equilibrium was made prior to the beginning of the therapeutic period and immediately preceding each subsequent administration of radioactive phosphorus. All dosage is referred to in intravenous equivalents; the effective oral dose is estimated as approximately 75 per cent of the same intravenous dose, the oral route having been used on occasion. Of the eleven patients selected for study, six had received no previous therapy, and five had had previous x-ray therapy. Each biweekly dose was determined as a function of the tolerance of the individual patient, measured in terms of abrupt thrombocytopenia or by other less acute evidences of bone marrow hypoplasia.  $P^{32}$  therapy was discontinued and other measures substituted in every case when an increase in the activity of the disease was indicated by additional lymphadenopathy or clinical evidence of bone involvement demonstrable by x-ray.

Parallel studies made in this clinic in an attempt to evaluate the therapeutic

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TABLE I

CASE	AGE (YR.)	SEX	DURATION OF SYMPTOMS BEFORE P <sup>32</sup> ADMINISTRATION	DURATION OF P <sup>32</sup> THERAPY IN WEEKS	BIWEEKLY DOSE IN MILLICURIE	PREVIOUS X-RAY THERAPY	FAVORABLE RESPONSE TO X-RAY AFTER CESSATION OF PHOSPHORUS THERAPY
1	35	F	10 yr.,	21.8	2.27	+	0
2	31	F	1 yr., 2 mo.	13.8	3.01	+	+
3	25	M	5 yr.	14.8	2.22	+	+
4	22	F	1 yr., 11 mo.	13.8	2.48	+	+
5	67	F	3 yr., 10 mo.	21.0	2.26	+	+
6	35	F	7 mo.	21.0	2.55	0	+
7	26	F	1 yr., 1 mo.	28.0	2.77	0	+
8	18	M	2 yr.	10.0	2.49	0	- (Died)
9	39	M	1 yr.	12.7	2.26	0	-
10	29	F	Uncertain	37.0	1.26	0	+
11	46	M	2 yr.	14.8	2.12	0	+
Average value	33.9	7 F 4 M	2 yr., 10 mo.	18.97	2.31	5 positive 6 negative	8 positive 1 negative

TABLE II\*

	DECREASE (CASES)	INCREASE (CASES)	UNCHANGED (CASES)
Sedimentation rate	5	5	1
Hematocrit	8	2	1
Hemoglobin	8	3	0
Red blood cells	7	3	1
Platelets	7	2	2
White blood cells	11	0	0
Lymphocytes	6	0	5
Monocytes	6	1	4

\*Represents a comparison of hematologic values obtained at the beginning and at the end of radioactive phosphorus therapy.

effectiveness of radioactive phosphorus in leucemia, multiple myeloma, metastatic carcinoma, lymphosarcoma, and erythremia will be presented elsewhere.

#### RESULTS AND DISCUSSION

In a majority of the eleven patients (Tables I and II) who were treated with radioactive phosphorus, a fall in the hematocrit, in the hemoglobin level, and in total red blood cells, platelets, and total white blood cells, including lymphocytes and monocytes, was recorded during therapy. These changes are similar to those observed by Hemplemann and co-workers<sup>2</sup> in patients with various hematologic dyscrasias who were treated with radioactive phosphorus. There were only two in the series of eleven patients whose sedimentation rates returned to within physiologic limits during therapy (Tables I and II). The most marked and abrupt decline in hematologic values was that recorded in the case of circulating platelets; a platelet decrease from normal or above to a level of 100,000 or less in ten patients, and 50,000 or less in six patients was observed.

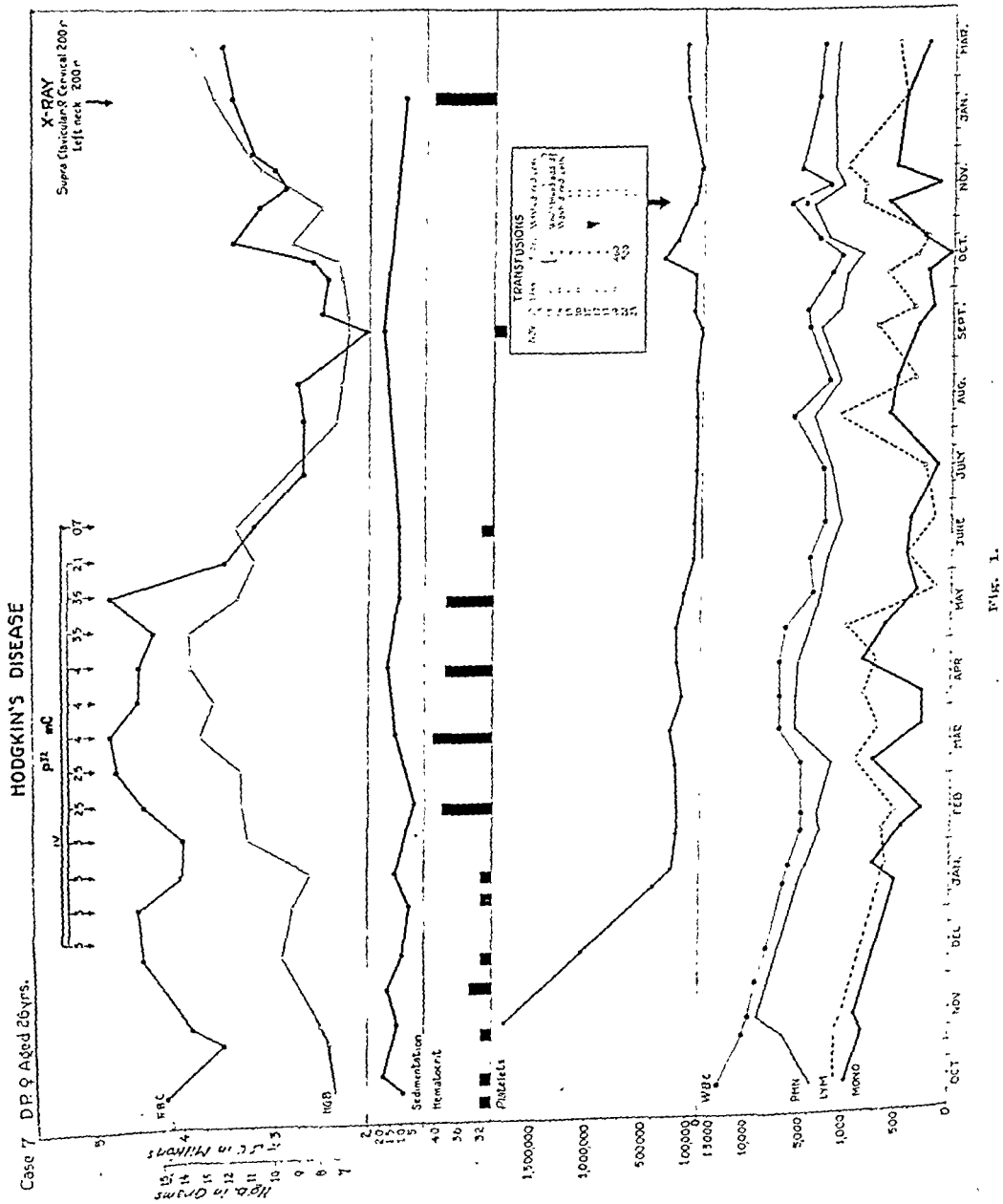
The average biweekly intravenous dose of phosphorus was 2.31 millicuries, the minimum being 1.26 millicuries and the maximum being 3.01 millicuries (Table I). Ten of the eleven patients treated developed additional adenopathy or demonstrable evidence of bone involvement during the course of therapy. The maximum and minimum periods during which treatment occurred, before additional evidence of disease activity developed, were thirty-seven weeks and ten weeks, respectively (Table I). One patient (Case 9, Table I) did not return for treatment after the eleventh week. Another patient (Case 8, Table I) died during therapy. The duration of temporary benefit occurring in a few patients cannot be correlated with the duration of the patient's illness at the time of treatment but is probably a function of the rate of activity of the disease at that

time. Eight of the nine patients who received x-ray radiation after cessation of phosphorus isotope therapy responded favorably to the former, as indicated in Table I.

It should be noted that in our experience platelet depression and other signs of marrow hypoplasia did not disappear in most cases after cessation of therapy. Despite the prolonged and severe platelet depression after cessation of  $P^{32}$  therapy, no clinical evidence of hemorrhage occurred.

Three representative cases (Figs. 1, 2, and 3—Cases 2, 6, and 7) have been chosen for illustration since they indicate graphically the general trends in hematologic values observed in the group of eleven patients studied.

CASE 7 (Fig. 1).—D. P., a 26-year-old woman, was first seen Aug. 8, 1943, giving a history of progressive and painless left cervical adenopathy of eight months' duration. There was also a history of weight loss of thirty-five pounds, lumbar backache increased during



menstrual periods, and an increase in nervousness for one year and insomnia, palpitation, weakness, and easy fatigue for six months. The patient had experienced hives (cause unknown) sporadically since childhood and stated that her paternal grandmother had allergic asthma for many years. Physical examination revealed enlarged tonsils and nontender inferior cervical adenopathy 2 by 2 cm. On palpation the enlarged nodes were moderately hard and somewhat adherent to the surrounding tissues. Laboratory studies revealed: White blood cell count 13,350; red blood cell count, 4.34 million; hemoglobin, 11 Gm.; hematocrit, 35; sedimentation rate, 1.3 mm. per minute (Rourke and Ernestine modification of Wintrobe method). The leucocytic differential was as follows: polymorphonuclear neutrophils, 65; polymorphonuclear eosinophiles, 9; lymphocytes, 19; monocytes, 7; platelets, 1,003,920; and reticulocytes, 1.8 per cent. Urinalysis and serologic tests for syphilis were negative. Biopsy and histologic study of a lymph node obtained from the left inferior cervical region confirmed the clinical diagnosis of Hodgkin's granuloma.

On Nov. 10, 1943, a tonsillectomy was done and the tonsils contained no evidence of Hodgkin's disease either macroscopically or microscopically.

On December 22 radioactive phosphorus was begun as noted in Fig. 1. During the course of  $P^{32}$  therapy there was an apparent slight decrease in the left inferior cervical adenopathy two or three weeks after treatment was started. There was, however, no subjective improvement and during the month of April a persistent elevation of temperature of 99° F. was noted.  $P^{32}$  was discontinued June 12, 1944, when it was discovered that additional adenopathy in the left superior cervical chain of nodes had developed.

On Oct. 10, 1944, the patient entered the hospital with a complaint of vaginal bleeding of one week's duration. Following a dilation and curettage, bleeding stopped promptly. A diagnosis of functional uterine bleeding was made and multiple washed red blood cell transfusions were given.

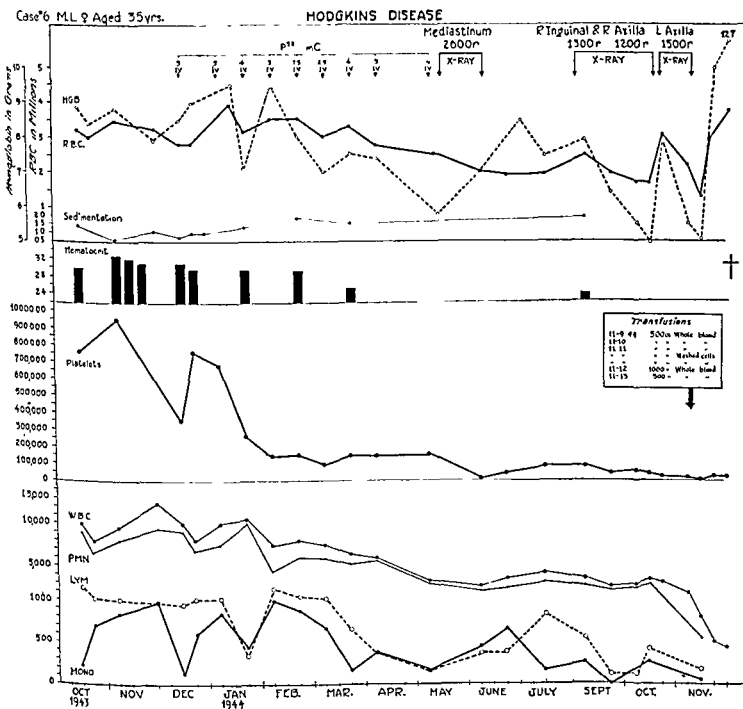


Fig. 2.



CASE 6 (Fig. 2).—M. L., a 35-year-old woman, was first seen Aug. 25, 1943, with a chief complaint of painless right cervical adenopathy of four months' duration. There were no abnormal subjective symptoms and no other contributory historical findings. Physical examination revealed no abnormality other than a nontender right inferior cervical adenopathy  $1\frac{1}{2}$  by  $1\frac{1}{2}$  cm. Laboratory studies revealed the following: white blood cell count, 7,300; red blood cells, 3,600,000; hematocrit, 38; and corrected erythrocyte sedimentation rate, 0.7 mm. per minute. Urinalysis and serologic tests for syphilis were negative. During the next four months the white blood cell count and sedimentation rate rose, the patient became more anemic, and itching of the skin and continuous fever became manifest.

On December 22 radioactive phosphorus therapy was begun as indicated in Fig. 2. On April 25, after four months of treatment and slight subjective improvement in symptoms, a large mass extending from the mediastinum into the upper left chest was observed by x-ray, and an afternoon temperature of  $102^{\circ}$  F. was recorded. Radioactive phosphorus was discontinued and x-ray therapy begun. The amount and duration of treatment are indicated in Fig. 2.

On October 4 there was a transitory drop in temperature and an increase in the size of the existing nodes. During early November, 1944, the patient developed fluid in the left pleural cavity and despite repeated drainage, rapidly became weaker and died November 15. A profound state of bone marrow exhaustion existed at the time of death. The apparent terminal rise in red blood cells and hemoglobin was produced by multiple transfusions of washed red blood cells.

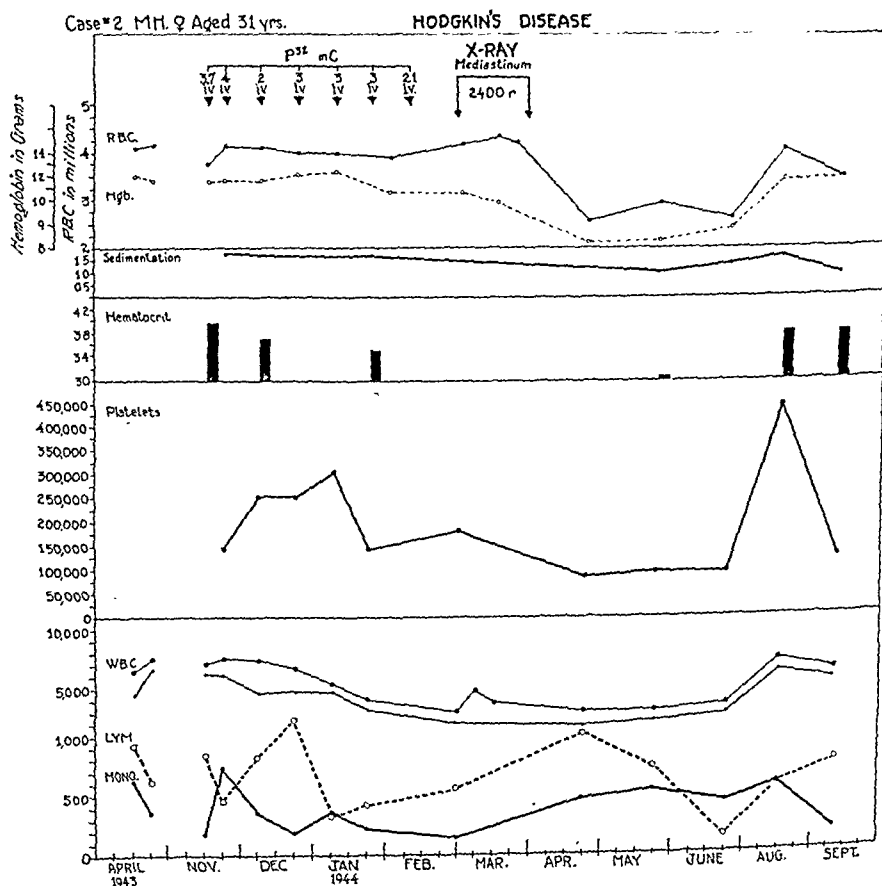


Fig. 3.

CASE 2 (Fig. 3).—M. H., a 31-year-old woman, first seen Jan. 21, 1943, gave a history of having had right cervical adenopathy seven years previously (1936). The adenopathy present at that time was said to have been accentuated by the removal of "infected tubes." Because of the recent change in node size, a biopsy was done and a diagnosis of Hodgkin's disease was made. As a result of this finding, an unknown amount of roentgen therapy was

given over the right cervical region. Oophorectomy, biopsy, and x-ray treatment were carried out elsewhere and complete data regarding them are not available. A recurrence of lymph node enlargement did not occur until six years later (August, 1942), at which time a mass was noted in the lower mid-cervical region. The patient began to complain of weakness, fatigue, nervousness, and numbness in both arms. It was the opinion of the surgeon in charge that the mass was due to thyroid enlargement. At operation (January, 1943) the enlargement was found to consist entirely of Hodgkin's tissue, and following a histologic diagnosis of Hodgkin's granuloma, the patient was referred to this clinic for therapeutic management. A few days after the operation, an eruption with many of the characteristics of erythema nodosum appeared on the lower legs and ankles and disappeared again after a few weeks. Hematologic studies were recorded as follows: hematocrit, 41; sedimentation rate, 1.1 mm. per minute (corrected); white count, 10,500; red cells, 4,110,000; hemoglobin, 11.7 Gm.; supravital differential: polymorphonuclear neutrophils, 63; polymorphonuclear basophiles, 2; polymorphonuclear eosinophiles, 5; lymphocytes, 19; monocytes, 11.

On Nov. 18, 1943, radioactive phosphorus administration was begun as indicated in Fig. 3. At this time there were palpable a few small left supra-clavicular nodes and a mass 1 by 1 cm. to the right of the sternum and attached to the third rib. One month after phosphorus isotope therapy was started, the patient complained of itching of the skin; after two months of  $P^{32}$  administration the patient complained of a burning sensation within the mass to the right of the sternum after each dose of phosphorus was given. After three months of treatment the patient developed a severe cold after which a noticeable increase in the external chest mass occurred and right lateral displacement of the trachea was noted. A few days later difficulty in swallowing became a prominent symptom. Radioactive phosphorus was discontinued and roentgen ray therapy was instituted as indicated in Fig. 3. There was definite clinical improvement noted and the external thoracic mass could no longer be felt, following the completion of x-ray therapy.

#### SUMMARY AND CONCLUSIONS

The use of radioactive phosphorus in the treatment of eleven selected patients with Hodgkin's disease in the dosage and under the conditions described has not proved of therapeutic value. Depression of hemocytopoiesis, with special emphasis on thrombocytopenia, has been noted.

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# THE RELATIONSHIP BETWEEN DIET AND THE MECHANISMS FOR DEFENSE AGAINST BACTERIAL INFECTIONS IN RATS

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## INTRODUCTION

THE importance of diet in resistance to infectious diseases has long been recognized. A number of controlled animal experiments has shown that such a relationship exists. Watson<sup>1</sup> found a greater number of fatalities in mice fed on a diet from which dried skimmed milk was omitted than in animals whose diet included the milk. Watson, Wilson, and Topley<sup>2</sup> then observed a higher susceptibility to natural contact infection in mice on a diet similar to this than in the animals that were well nourished. The importance of nutrition in nonspecific resistance to infection was clearly demonstrated in mice by Church.<sup>3, 4</sup> Dietary protein has more recently been found by Sako<sup>5</sup> to play an important role in the susceptibility of mice to inoculation with a virulent culture of pneumococci as measured by the postinoculation survival time. Carbohydrates and fats had little influence on this susceptibility. Cannon, Chase, and Wissler<sup>6</sup> depleted rabbits of proteins by restricting the intake and by plasmapheresis and obtained lower agglutinins than in normally fed animals. This was especially true in young animals. Thus, Cannon has emphasized<sup>7</sup> the importance of acquired immunity in resistance to microbial agents and has correlated<sup>8</sup> hypoproteinemia with susceptibility to intercurrent infections in human beings. In Alabama, Riddle, Spies, and Hudson<sup>9</sup> studied 150 patients with pellagra who had definite clinical lesions. *Staphylococcus aureus* and *Streptococcus hemolyticus* were isolated from cheilotic and ocular lesions, and these organisms disappeared following riboflavin therapy. In gingivitis and stomatitis large numbers of Vincent's organisms were found in addition to the bacteria mentioned; these also disappeared following nicotinic acid treatment. In acutely deficient patients a low complement titer and a reduced bactericidal activity of the blood were found. As the dietary improved, these defense mechanisms were returned to normal levels.

Numerous papers in this field have dealt with the relationship of single vitamin deficiencies to specific defense mechanisms. In studies on blood, Abbott and Ahmann<sup>10</sup> have reported a decrease in total white blood cells and in polymorphonuclear cells in rats, and Abbott, Ahmann, and Overstreet<sup>11</sup> found a similar decrease in leucocytes in human beings with clinical vitamin A deficiency. Crimm and Short, on the other hand, found only a neutrophilic index lag in vitamin A-deficient rats<sup>12</sup> and dogs.<sup>13</sup> In cases of pulmonary tuberculosis given large doses of vitamin A, five of ten patients had a left shift and the remainder a right shift.<sup>14</sup> In none of their work did they observe a decrease in total number of white blood cells. Verder and Petran<sup>15</sup> in their study of vitamin A-deficient monkeys observed a slight increase in the average number of leucocytes,

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polymorphonuclear leucocytes, and erythrocytes. In ten human beings maintained on an A-deficient diet for 188 days, Wagner<sup>16</sup> found a marked leucopenia with a preponderance of degenerate myeloid cells. There was also a thrombocytopenia and a decrease in hemoglobin and erythrocytes.

In 1935, Day, Langston, and Shukers<sup>17</sup> found that the rhesus monkey developed a fulminating fatal blood disease characterized by leucopenia and anemia when fed a diet deficient in B<sub>12</sub> (G). Ulceration of the gums was a consistent accompaniment of the hypocythemia, and diarrhea was common. Several papers have since appeared,<sup>18-20</sup> and it is known that yeast and liver fractions either prevent or cure this disease. Studies on rats fed diets containing from 1 to 2 per cent of one of several sulfa drugs show that severe leucopenia and sometimes anemia may result.<sup>21-25</sup> It can be cured with liver fractions,<sup>21, 23, 24</sup> folic acid concentrates,<sup>21</sup> and crystalline folic acid<sup>23</sup> and improved but not cured by biotin and xanthopterin.<sup>25</sup> The sulfa drugs in nutritional studies are used to alter the intestinal flora of the animal, but results due to toxicity may complicate the natural picture. For this reason the work of György, Goldblatt, Miller, and Fulton<sup>26</sup> and Shukers and Day,<sup>27</sup> in which malnutrition alone has been shown to produce leucopenia in rats, is of special importance. György and co-workers used a diet deficient in vitamin B<sub>12</sub> and found that the leucopenia, thrombocytopenia and anemia which developed could not be cured with vitamin B<sub>12</sub> alone but required Peter's eluate. Shukers and Day fed the animals a riboflavin-deficient diet. Both the deficient rats and the inanition controls became leucopenic, but with the latter group the condition was more severe. Nutritional leucopenia and ulcerative stomatitis in dogs fed a blacktongue-producing diet is reported by Miller and Rhoads.<sup>28</sup> In patients suffering from buccal ulcers a granulopenic leucopenia is frequently encountered.<sup>29</sup>

A reduced phagocytic activity has been reported by Gellhorn and Dunn<sup>30</sup> in undernourished dogs after from six to eight days' starvation. They compared<sup>31</sup> the ingestion of starch granules by washed leucocytes suspended in the experimental serum of each of fifty-seven vitamin A-deficient rats with the ingestion of granules by twenty-two control animals. Thirty of the deficient animals had decreased phagocytosis, and sixteen had increased and eleven had the same phagocytic activity. The severity of the deficiency symptoms did not parallel the changes in phagocytic index. Cottingham and Mills<sup>32</sup> found a decrease in *in vitro* phagocytic activity of rat granulocytes in animals fed diets with single vitamin deficiencies. This was true for thiamine, riboflavin, pyridoxine, pantothenic acid, choline, singly, and for vitamins A and D combined. No inanition controls were used, but Cottingham and Mills interpreted their results as being dependent upon the general nutritional level of the animals rather than to the effects of the vitamins *per se*.

The humoral defense mechanism of animals on various types of nutritional regimes has received the attention of several investigators. The tissues of rats deficient in vitamin A were found by Prickett, Miller, and McDonald<sup>33</sup> to have excess lysozyme. In agreement with this observation is the work of Sullivan and Manville,<sup>34</sup> in which the colon of rabbits deficient in vitamin A was found to have four times the lysozyme content as that of controls. In the most comprehensive report of studies on human beings, Feller, Roberts, Ralli, and Francis<sup>35</sup> kept subjects on diets adequate in all respects except for either vitamin A or vitamin C for periods of six, fourteen, nineteen, twenty, or twenty-six weeks. They could find, under these conditions, no influence on the capacity of nasal secretions to inactivate influenza virus, the titer of neutralizing antibodies

for influenza virus, the activity of lysozyme in nasal secretions, complement titer in blood serum, or phagocytic activity for pneumococci of leucocytes in heparinized blood. Negative results were also obtained by Natvig<sup>36</sup> in experimental animals deprived of vitamins A, B, C, or D. He tested, without finding any change, the complement titer, bactericidal power of the blood for dysentery bacilli or *Staph. aureus*, the ability to form agglutinins, and the phagocytic activity. Scaglione<sup>37</sup> has reported a reduction in the agglutinin titers against typhoid and paratyphoid in infants and children deficient in vitamin A. Madison, Fish, and Frick<sup>38</sup> found that nicotinic acid mixed in varying amounts with the antigenic doses of horse serum lowers the precipitin titer in rabbits in small amounts and raises it when given in large amounts. A slight retardation in building up agglutinins against typhoid vaccine was found by Tecce and Visconti<sup>39</sup> in rabbits given nicotinic acid in excess. Juszat,<sup>40, 41</sup> in a series of experiments with rabbits fed a vitamin-free diet, reports a lowered bactericidal power in blood and a 90 per cent reduction in horse serum precipitins. Vitamins A and D and dried brewers' yeast failed to alter the response. Rose and Kolmer<sup>42</sup> found no change in the complement titer of dogs after four months of B avitaminosis and anhydremia. In fifty persons with vitamin B deficiencies, Morey and Spies<sup>43</sup> showed that the agglutinin titers to *Bacterium tularensis* were lower and less well maintained than in normal controls.

Single vitamin deficiency studies are important in elucidating the metabolic function of the vitamins, but single deficiency states seldom occur naturally. For this reason, it was considered of interest to test the various defense mechanisms against infectious diseases in rats fed a diet similar to that upon which persons in an area of endemic malnutrition develop mixed deficiencies, a diet which has been shown to be deficient in many nutrients.<sup>44</sup> Accordingly, a diet for rats was devised, based on the foods eaten by a family studied at the Nutrition Clinic of the Hillman Hospital in Birmingham and characteristic of the diet of many families in this area. It was first used experimentally by Bieler, Johnson, Grant, and Spies,<sup>45</sup> who found that rats suffered loss of weight, loss of appetite, and alopecia, and that normal development was obtained only after the diet had been supplemented with high-grade protein, vitamins, and minerals. Various combinations of the three supplements were found to support growth above the level of the basal diet but considerably below the control level. For this study the supplements which were found to give the best intermediate stages of growth between the basal diet and the diet with all essential supplements were selected from among those used by Bieler and associates. This was done to determine whether resistance to disease could be graded from low to high by dietary means. The results reported here show that this can be done only when the animals are reasonably well nourished and that weight alone is insufficient for predicting the susceptibility to disease.

#### METHOD

One hundred Sprague-Dawley weanling rats were divided into ten groups, with ten rats in each group. Five groups were males and five groups females. The rats were housed five to a cage. The cages were kept in a steam-heated room, and all animals were treated as uniformly as possible except for diet. They were fed as follows:

Groups 1 and 6	Basal
Groups 2 and 7	Basal + casein
Groups 3 and 8	Basal + casein + minerals
Groups 4 and 9	Basal + casein + B vitamins
Groups 5 and 10	Basal + casein + B vitamins + minerals

The animals in Groups 1 to 5, inclusive, were males, and those in Groups 6 to 10, inclusive, were females. The basal diet consisted of 35.6 per cent corn meal, 28.1 per cent unenriched white flour, 17.6 per cent pork fat, and 18.7 per cent cane sugar. These were thoroughly mixed with sufficient water to make the ration more easily eaten. Eighteen per cent vitamin-free casein\* was added to all diets except that for the animals in Groups 1 and 6. The mineral supplement was composed of a mixture of the following salts,<sup>46</sup> which constituted 4 per cent of the diet in Groups 3, 5, 8, and 10:

	GM.
Calcium carbonate	600
Potassium acid phosphate	645
Calcium acid phosphate	124
Magnesium sulfate	204
Sodium chloride	335
Ferrie citrate	55
Potassium iodide	1.6
Manganese sulfate	10
Zinc chloride	0.5
Copper sulfate	0.6

The synthetic B vitamins were dissolved in distilled water and added to the other ingredients which then offered the following amounts per day per rat:

	MG.
Thiamine	0.2
Riboflavin	0.2
Pyridoxine	0.2
Inositol	0.2
Ca pantothenate	10.0
Nicotinamide	25.0
Choline	200.0

All the animals received an ad lib quantity of food and the daily consumption for those in each cage was determined by weight difference. This, however, was not an accurate measure since some loss from scattering occurred. Each animal was weighed twice weekly and the average weight per group was calculated. The animals were placed on the diets Sept. 15, 1944, and the final tests were completed Feb. 7, 1945, a total of 146 days. Throughout this time, all rats survived except those lost as a direct result of experimental procedures. There were, therefore, no deaths directly attributable to malnutrition.

A measure of the effectiveness of the three defense mechanisms subject to experimental tests was undertaken. This includes a determination of the total number of leucocytes with differential counts, the phagocytic activity of the granulocytic leucocytes, and the ability to produce antibodies, agglutinins. The only important factor omitted in these experiments is the barrier to invasion by infective agents offered by the skin and mucous membranes. The significance of this barrier in health and disease is not to be minimized, but unfortunately at the present time there is no technique known to us that permits a quantitative estimation of its activity alone. Stryker and Janota<sup>47</sup> found no change in the permeability of the intestinal wall to *Salmonella enteritidis* in vitamin A-deficient rats, but their procedure does not exclude the role of phagocytes.

The tip of the tail was cut in order to obtain blood for erythrocyte counts and white cell differential counts. Bureau of Standards pipettes and counting chambers were used. Wright's stains were made for the differentials. Counts were made on five rats in each of the ten groups.

Phagocytic activity was determined in vitro by the technique of Cottingham and Mills.<sup>32</sup> One-half cubic centimeter of heart blood is diluted in the same

\*S. M. A. Corporation, Chicago, Ill.

volume of heparinized\* saline and then 0.2 c.c. of a standardized suspension of *Micrococcus candidus* in saline is added. The tube is gassed with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide and shaken for four minutes in a water bath at 38° C. A blood smear is made immediately at the end of the period, stained with Wright's stain, and the number of bacteria is counted in sixty neutrophilic granulocytes. The phagocytic activity of the cells in the five rats in each of the ten groups not used in making blood counts was measured. The final value for each group is thus calculated from a total of 300 white blood cells.

In order to eliminate a possible variation in phagocytosis with time, the blood was withdrawn from all the rats on the same day. Errors that might be attributed to a change in the density of the bacterial suspension due to standing at room temperature were overcome by testing the blood of one rat from each of the five groups of males at the same time and repeating until all had been tested. This was continued without delay throughout the morning hours, and the same procedure was followed in the afternoon for the five groups of females. An aliquot of the same bacterial suspension was refrigerated throughout the morning hours and was then brought to room temperature for use in the afternoon. No consistent variation in phagocytic activity of cells from rats in the same group occurred. Once the smears are stained, the actual microscopic counts can be made over a period of days.

The same rats used for blood counts were originally selected for testing agglutinin production. A phenolized culture of *Bact. tularensis* with a density of 7.3 billion cells per cubic centimeter was employed as the antigen.† The animals were injected intraperitoneally at three-day intervals until a total of four injections was given. The first two doses were 0.1 c.c. of vaccine and the last two doses were 0.15 c.c., a total of 0.5 c.c. Two weeks after the final injection, and at biweekly intervals for eight weeks, the males and females were alternately tested for agglutinin titer, but the titers remained very low throughout this time. The serum of the animals in Groups 5 and 10 showed slight agglutination in only the second tube (1:40). Therefore, since this particular antigen gave such a low titer, it was considered desirable to repeat the experiment with a different vaccine.

The five rats most uniform in size that remained in each of the ten groups were placed together in one cage. Each rat was inoculated subcutaneously with 0.2 c.c. mixed typhoid-paratyphoid vaccine.‡ The injections were repeated at intervals of five days until a total of three inoculations was given. Ten days after the immunizing doses had been completed, heart blood was withdrawn and the serum agglutinin titer was determined with the same lot of vaccine for the antigen.

#### RESULTS

The animals were permitted to remain on the diet for two months before any tests were begun. During this period their weights were checked twice weekly and the growth curves for each group are shown in Fig. 1. After eighty days heart punctures were begun in order to obtain blood for the agglutinin titers, and therefore the death of animals in some of the groups made further records inaccurate. After two months on the diets the animals were photographed, and

\*The heparin used in this work was supplied by Hoffmann-La Roche, Inc., Nutley, N. J.

†Supplied by Dr. Lee Foshay, Professor of Bacteriology, College of Medicine, University of Cincinnati.

‡Lederle Laboratories, Inc., Pearl River, N. Y.

their appearance at this time is shown in Fig. 2. There was a subsequent increase in alopecia in the rats in all groups, except those in Groups 5 and 10, but otherwise there was no great change in the general physical characteristics of the animals. A more detailed description of rats fed on these diets may be found in the paper by Bieler, Johnson, Grant, and Spies<sup>45</sup> now in preparation.

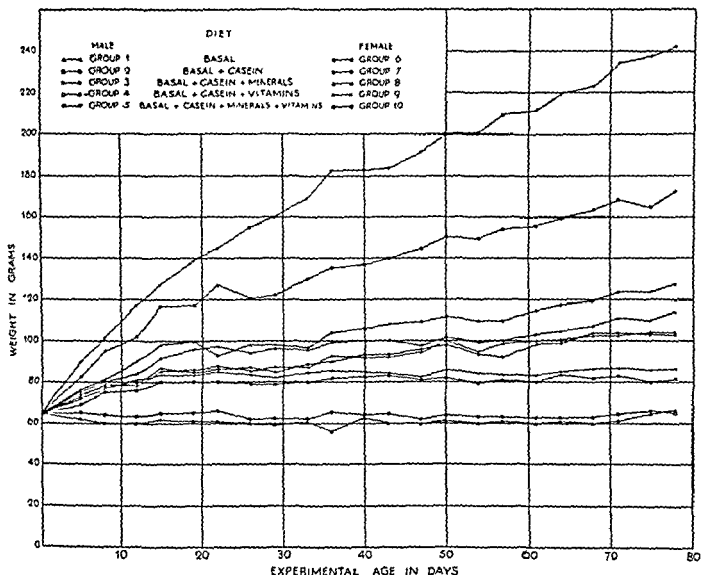


Fig. 1.—Growth curves of rats by groups. Each curve is the average of ten animals. Beginning age is 28 days.

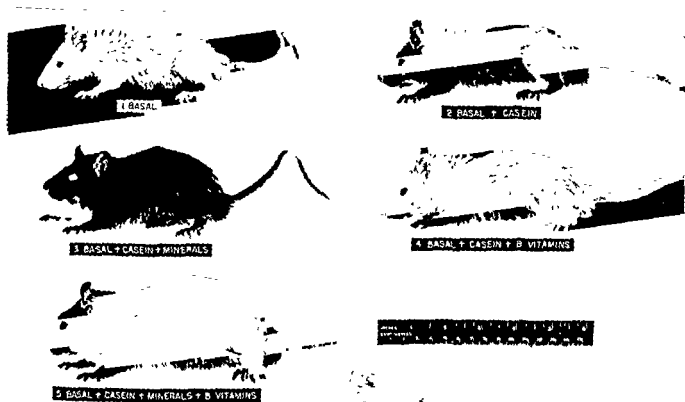


Fig. 2.—Photographs of males from each of the five groups after eighty days on the respective diets. All animals are the same age.



1. *Blood Studies.*—In Table I are summarized the results of the blood studies. Blood counts from the rats in Groups 1 and 6 were obtained after sixty-one days on the diet, and on those in the remaining groups in order (2 and 7, 3 and 8, etc.) during the next ten days until completed. The animals on the basal diet, both males and females, had total white cell counts only about one-third of those in the well-fed, normal groups. The remainder of the groups fell within the normal range for the rat<sup>48</sup>; however, they were all lower than the adequately fed animals. It is interesting to note that the differential leucocyte picture is shifted toward a relative increase in polymorphonuclear neutrophils with a corresponding relative decrease in lymphocytes and an absolute decrease in all cellular types. This is in agreement with the results reported by Shukers and Day<sup>27</sup> in their blood studies on rats fed a riboflavin-deficient diet with inanition controls. The latter were more severe. No change was observed in the red blood cell totals within this period.

TABLE I. BLOOD STUDIES OF RATS ON DIFFERENT DIETS

GROUP	R. B. C. (IN MILLIONS)		W. B. C.		P. M. N.		LYMPHO.		P. M. E. (PER CENT)	MONO. (PER CENT)
	AVER-AGE	RANGE	AVER-AGE	RANGE	AVER-AGE (PER CENT)	RANGE (PER CENT)	AVER-AGE (PER CENT)	RANGE (PER CENT)		
1	8.24	7.82-8.59	5,963	5,100-6,650	28.5	20-34	68	62-75	0.5	3.0
2	8.875	8.10-9.90	9,150	6,800-13,000	34	26-48	65	51-73	0	1
3	7.96	7.15-8.95	8,430	6,300-11,350	36	21-44	62	53-74	1	1
4	9.431	9.05-9.775	14,725	12,800-19,450	43	35-49	54	49-62	1	2
5	8.525	8.025-9.325	15,250	12,250-18,500	18	13-22	76	71-81	3	3
6	8.88	7.60-10.45	4,980	2,100-6,550	29	20-36	67.5	60-78	0.5	3
7	9.24	8.65-9.55	10,810	8,600-13,050	34	23-44	63	53-74	1	2
8	8.44	7.75-9.325	7,640	5,000-9,300	25	15-45	72	54-81	1	2
9	8.705	8.05-9.125	11,810	7,700-15,750	27	17-37	70	60-78	1	2
10	8.636	7.60-10.15	14,216	13,000-17,750	21	17-27	75	66-81	2	2

2. *Agglutinin Production.*—In the agglutinin tests against *Bact. tularensis* one rat in Group 1 had a titer of 1:320 and one rat in Group 8 had a titer of 1:160. All the remaining animals failed to give complete agglutination in the first tube and showed none at all beyond the third (1:80). The lack of uniformity, the very low titers obtained, and the slowness of response to the antigen made it desirable to test the antibody formation with a different vaccine.

In Table II are shown the results of this study with the typhoid-paratyphoid vaccine. The basal male rats had titers 23.3 per cent as high as the well-fed males, and the females were 45.1 per cent of normal. The three intermediate groups were also greatly inhibited in their ability to form antibodies. There was a spread in the individual titers of rats in each group, but in no case did a deficient rat have a titer as high as the controls. Conversely, the control rats had a more uniform titer, and their lowest titer was equal to, or above, the highest titer of any of the deficient animals.

TABLE II. AGGLUTINATION TITERS OF RATS FED DIFFERENT DIETS

GROUP	AVERAGE TITER	RANGE
1	1:2390	1:320-1:5120
2	1:3718	1:640-1:5120
3	1:2810	1:1280-1:5120
4	1:4120	1:2560-1:5120
5	1:10,240	1:10,240-1:10,240
6	1:7368	1:5120-1:10,240
7	1:6656	1:2560-1:10,240
8	1:7168	1:2560-1:10,240
9	1:9216	1:5120-1:10,240
10	1:16,336	1:10,240-1:20,480

3. *Phagocytic Activity*.—The second column in Table III shows the mean number of bacteria engulfed by neutrophilic polymorphonuclear leucocytes in all rats in each group. These cells in rats on a basal diet were 60.6 per cent for the males and 64.2 per cent for the females as active as those in the control groups. The rats of Groups 2 and 7 and 3 and 8 did not vary significantly from

TABLE III. PHAGOCYTIC ACTIVITY OF ANIMALS ON DIFFERENT DIETS

GROUP	MEAN NUMBER OF BACTERIA PHAGOCYTIZED PER CELL	DIFFERENCE OF MEANS FROM BASAL GROUP	PROBABLE ERROR OF DIFFERENCE $\times 4$
1	8.9400 $\pm$ 0.3554		
2	8.2666 $\pm$ 0.3719	0.6734	2.0568
3	7.8866 $\pm$ 0.3534	1.0534	2.0048
4	11.9400 $\pm$ 0.3677	3.0000	2.1037
5	14.7400 $\pm$ 0.4235	5.8000	2.2114
6	15.9934 $\pm$ 0.2459		
7	14.7500 $\pm$ 0.2750	1.2374	1.428
8	14.3320 $\pm$ 0.4004	1.6614	1.8796
9	19.0784 $\pm$ 0.1566	3.0850	1.1663
10	23.3466 $\pm$ 0.3057	7.3532	1.5693

the basal diet animals, while the rats of Groups 4 and 9 were within 74.2 and 81.7 per cent normal, respectively. Column 4 is the calculated probable error of the difference between means, times four. This shows how large the figures in column 3 must be before the difference in means is significant. There is thus no significant increase in phagocytic activity in any of the groups except 4 and 9 and 5 and 10.

The larger number of bacteria phagocytized by the females as compared to the males may or may not be significant. All procedures, as previously mentioned, were carried out on the same day and were designed so as to give a quantitative check between the males in the morning and the females in the afternoon. In spite of the precautions taken, a change in the density of the suspension might very well account for the difference in the results. It was not considered advisable to attempt to check the results by repeating the counts since Mills\* has found that bleeding the rats upsets the phagocytic activity so as to prevent a reliable check. Even so, it is interesting to note the close agreement between the percentage variation of the sex groups.

#### DISCUSSION

These studies support the working hypothesis that resistance to bacterial infections may be depressed by inadequate nutrition. Their importance is enhanced by the fact that the animals were eating the same diet that gives rise to the mixed deficiencies seen in patients at this clinic. The demonstration that antibody production is depressed in rats is in agreement with the observations of Morey and Spies<sup>43</sup> in pellagrins. Also, the fact that granulocytic leucopenia occurred in rats is interesting because for several years we have been studying the incidence of and role of nutrition in leucopenia in people in an area of endemic malnutrition. Furthermore, the lowered phagocytic activity in rats on an inadequate diet is being supported, at least in part, by studies now in progress on the *in vitro* phagocytosis of the granulocytes of malnourished persons.

It is evident from the fine work of Foster, Jones, Henle, and Dorfman<sup>44, 45</sup> and Rasmussen, Waisman, Elvehjem, and Clark<sup>51</sup> that vitamin deficiencies do not invariably lead to a lowered resistance to infectious diseases. These investi-

\*Personal communication.

gations show that with the virus of poliomyelitis, a thiamine-deficient mouse is actually more resistant than the well-nourished mouse. The time for onset of symptoms, their severity, and, to some extent, the mortality rate are all in accord with this fact. The explanation is at present not clear, but an attractive suggestion is that some nutrient essential for the reproduction of the virus is absent. Since the defense mechanisms against bacterial diseases do not seem to function in the same way against many virus diseases, the results reported in this paper need not be considered as contradictory to the above. On the other hand, since the difference in final mortality rate between the various groups of mice studied by Foster and associates is the least significant of all the criteria used in their study, the fatal outcome of the infection was therefore not so strikingly altered by diet. Moreover, the absence of clear-cut results in detecting a difference in agglutinin production with *Bact. tularensis* as antigen suggests that the procedure adopted for testing the influence of malnutrition on susceptibility to disease may be important.

The hypothesis that a well-balanced diet is important in maintaining the defenses against infections is supported by this work. Of the rats receiving the various dietary supplements employed in this study, only those (Groups 5 and 10) that received minerals, proteins, and vitamins were normal. Of the rats on the remaining diets, those of Groups 4 and 9 (protein and vitamins) showed a significant elevation of the defense level above that of the rats in the basal group but somewhat lower than the adequately fed rats. Thus, if minerals or vitamins are withheld, the various defenses may be depressed but not in the same proportion as growth. However, it seems unlikely that it is because of a specific functional requirement for such factors alone rather than the extent to which the absence of that factor influences the well-being of the whole organism. Therefore, in mixed deficiencies, the importance of restoring the organism to a balanced nutritional regime becomes apparent if that organism is to be able to defend itself adequately against the onslaught of bacterial infections.

#### SUMMARY

1. One hundred Sprague-Dawley weanling rats were divided into ten groups with ten rats in each group. The rats in five groups were males and those in the other five groups were females. One group from each sex was fed a basal diet composed of corn meal, unenriched flour, pork fat, and sugar in approximately the same proportion as is eaten by many families seen at the Nutrition Clinic, Hillman Hospital, Birmingham, Ala. The remaining four groups of males and of females were given the basal diet supplemented with casein, casein and minerals, casein and vitamins, and casein, minerals, and vitamins.

2. Growth was recorded and three defense mechanisms against bacterial infections were measured after the rats had been on the diets for a period of from 61 to 146 days.

3. There was a decided decrease in total leucocytes and in all leucocytic elements in the rats on the basal diet after two months. The differential counts showed a relative increase in granulocytes and a relative decrease in lymphocytes in the same animals. Only those rats receiving all three supplements were normal, while the others were intermediate between the two extremes.

4. The males and females on the basal diet had typhoid agglutinin titers only 23 per cent and 45 per cent, respectively, as high as the well-fed groups. Of the other groups, only those animals receiving casein plus vitamin supple-

ments showed an appreciable elevation above these values, and they were only about 50 per cent of normal.

5. The phagocytic activity gave results similar to those for antibody production. Only those animals with the casein plus vitamin supplement had values significantly approaching the activity of the well-fed rats. All other animals were only about 60-65 per cent as active.

6. These observations support the working hypothesis that resistance to certain bacterial infections may be depressed by inadequate nutrition and are in agreement with work previously done with patients. Malnutrition is thus shown to be a factor in susceptibility to infection, but this is not necessarily true in every type of infection.

We wish to acknowledge the assistance of Miss Carol Johnson for her aid in feeding, weighing, and caring for the animals throughout the period of this investigation. We are also grateful to Dr. C. A. Mills for granting us space in his laboratory for housing the animals, for the use of some of his equipment, and for his help and suggestions with the technique involved in making the phagocytic activity measurements.

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animals was entirely nontraumatizing and, as we have previously stated, simulated closely the manner in which human pneumococcal infections occur.

A total of eighty-two rats, each approximately 300 grams, was thus inoculated. Of these, seventy-seven animals (93.9 per cent) became infected as manifested by blood cultures positive for type I pneumococci. Only those animals developing positive blood cultures were used in this study.

Cultures of the blood were made just prior to the initial treatment, at twenty-four hours after inoculation, at forty-eight hours, and at seventy-two hours, just prior to succeeding treatments. They were then made at daily intervals until death ensued or until no growth was obtained. The blood for culture was plated on Avery's medium for pneumococci, to which was added 5.0 mg. per 100 c.c. of paraminobenzoic acid.

Determinations of free and conjugated sulfadiazine in the blood were made according to the procedure of Bratton and Marshall.<sup>7</sup> A Klett-Summerson photoelectric colorimeter with a No. 54 filter was used.

All animals used, whether dying spontaneously before the fifteenth day or sacrificed on the fifteenth day, were autopsied.

Although seventy-seven rats were successfully infected, seventy-four are included in this report, since in three animals the suspensions or solutions of the sulfonamide drug were introduced accidentally into the trachea, producing immediate death. There was, therefore, an unequal distribution of animals in some of the groups.

To insure accurate dosage, the compounds were either suspended or dissolved in distilled water and administered by gavage.

#### DOSAGE OF SULFADIAZINE AND SODIUM BICARBONATE

A series of nineteen animals, in which no therapy had been given, was used as a control for those receiving treatment. Therapy with sulfadiazine, sodium bicarbonate, and a combination of the two was begun twenty-four hours after inoculation with pneumococci.

Sodium bicarbonate, 500 mg. per animal, was administered to each of twenty animals on three successive days.

Sulfadiazine, 300 mg. of drug per animal, was given to each of sixteen animals on three successive days.

Sodium bicarbonate and sulfadiazine combined, 300 mg. of sulfadiazine and 500 mg. of sodium bicarbonate, was given to each of nineteen rats on three successive days.

#### RESULTS

*Series I (Control Animals).*—Sixteen of the nineteen rats in Series I died. The mortality rate was 84.2 per cent. The average period of survival of those dying spontaneously was 6.0 days. Of these, two had pneumonia alone. Eight had, in addition to pneumonia, one complication; meningitis occurred in two, empyema in four, peritonitis in one, and pericarditis in one. Three had two complications: empyema and pericarditis occurred in two, and pericarditis and peritonitis in one. In one, empyema, pericarditis, and meningitis, as well as pneumonia, were found.

Of the three animals surviving fifteen days and sacrificed, two had resolving pneumonia and a third had active pneumonia involving the left lung.

The average duration of bacteremia of the surviving animals was 5.6 days.

*Series II (Sodium Bicarbonate).*—Seventeen of the twenty animals in Series II died, giving a mortality rate of 85 per cent. The average period of survival of those dying spontaneously was five days. Of these, eight had pneumonia alone. Three had one complication in addition to pneumonia; two rats had pericarditis and one, peritonitis. Five had two complications: peritonitis and meningitis occurred together in two rats, and empyema and pericarditis in three. Meningitis, pericarditis, and empyema complicated the pneumonia in another of the rats that died spontaneously.

Of the three animals sacrificed, one had resolving bilateral pneumonia, and the others presented evidence of active bilateral pneumonia.

The average duration of bacteremia of those sacrificed was 6.3 days.

*Series III (Sulfadiazine).*—Only one of the sixteen rats in Series III died, giving a mortality rate of 6.2 per cent. This rat died on the eighth day with bilateral pneumonia and empyema, the blood stream had become sterile on the third day.

Of those sacrificed on the fifteenth day, autopsy revealed no pathology in two, resolving pneumonia in seven, fibrinous pleurisy in one, and bronchopneumonic involvement in five.

The average duration of bacteremia of those sacrificed was 4.25 days.

*Series IV (Sulfadiazine and Bicarbonate).*—There were nineteen rats in Series IV. Death occurred in nine, or 47.4 per cent. Four died on the fourth day, one on the sixth, three on the seventh, and one on the eighth, the average period of survival being 5.67 days. Five of those dying had pneumonia alone. In two, pneumonia was accompanied by one complication (empyema, peritonitis), and the other two rats each had a combination of empyema and pericarditis in addition to the pneumonic involvement. Hematuria occurred in the rat with pneumonia and peritonitis, this animal dying on the seventh day, and in two of the rats with pneumonia alone, both of which died on the fourth day.

Six of the animals sacrificed on the fifteenth day had resolving pneumonia, two had bronchopneumonic involvement, and one had no pathology.

The average duration of bacteremia of those sacrificed was 4.8 days.

#### SUMMARY AND CONCLUSIONS

1. Type I pneumococcal infection as manifested by positive blood cultures was produced in seventy-four albino rats. In those rats dying spontaneously, pneumonia and, in most instances, such complications as empyema, pericarditis, peritonitis, or meningitis were found at post-mortem examination.
2. Sodium bicarbonate, sulfadiazine, and sulfadiazine in combination with sodium bicarbonate were administered to sixty-six animals, treatment being started twenty-four hours after inoculation.
3. In Type I pneumonia in rats, the administration of sodium bicarbonate alone resulted in a mortality rate essentially the same as in those animals that received no medication at all. However, sodium bicarbonate in combination with sulfadiazine resulted in a mortality rate of over seven and one-half times that obtained with sulfadiazine alone.
4. In the rats receiving sodium bicarbonate alone and in combination with sulfadiazine, the cause of death was pneumonia with a short survival period, whereas in those receiving no sodium bicarbonate, the survival periods were longer and the animals died chiefly of complications.
5. The average duration of bacteremia of the sacrificed animals was longer

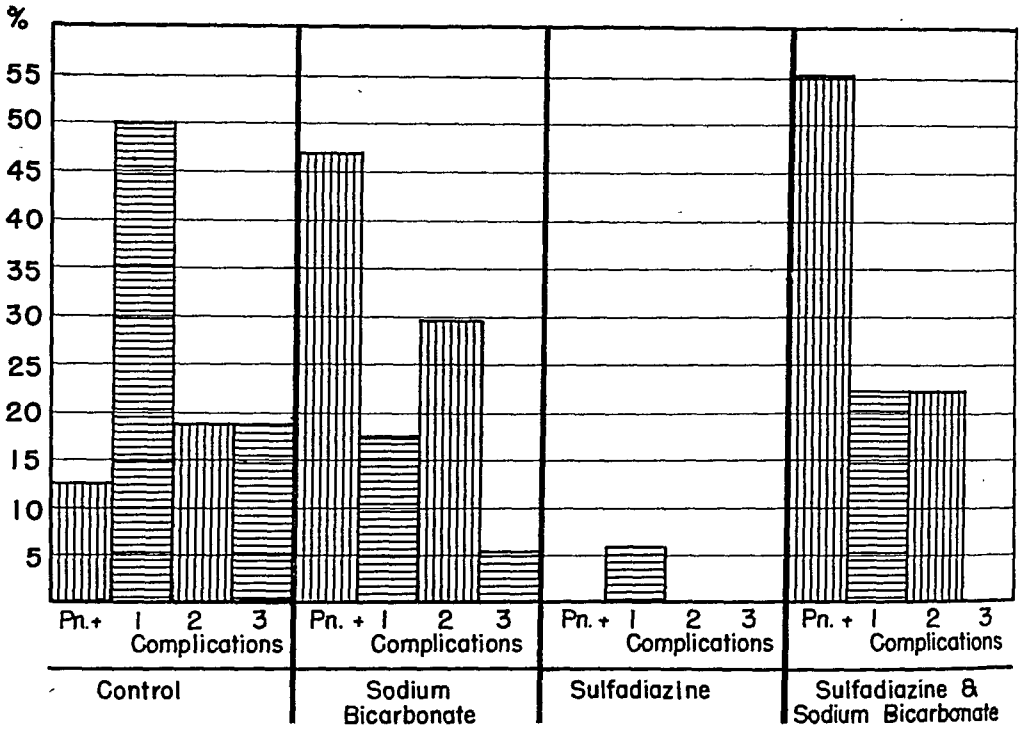


Fig. 1.—Per cent of total mortality due to pneumonia or to its complications. In each group are shown per cent of total deaths, in that group, due to pneumonia (*Pn.*), and to pneumonia with one complication (1) or with two (2) or three (3) complications. The complications were empyema, peritonitis, pericarditis, or meningitis.

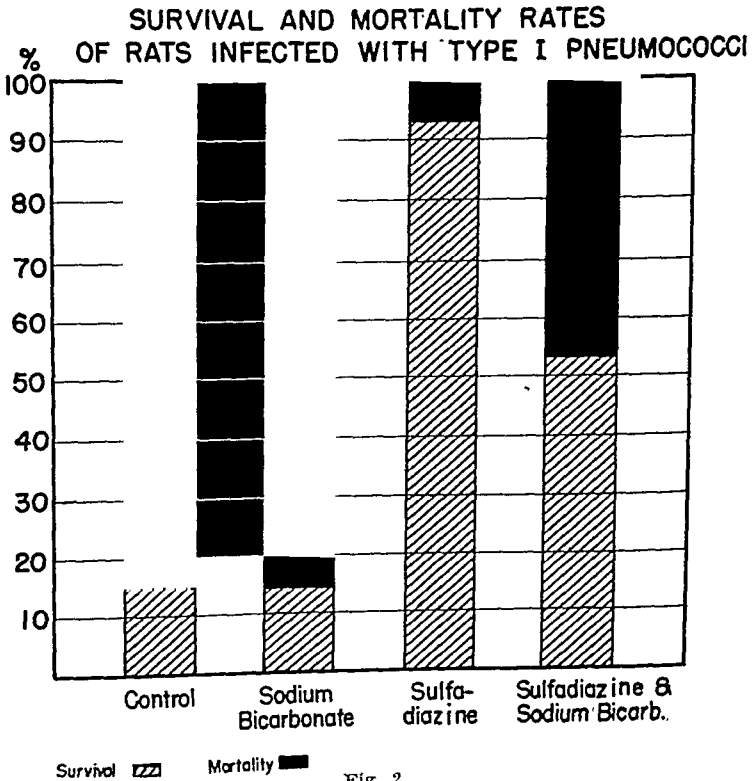


Fig. 2.

in the rats receiving sodium bicarbonate than in those in either of the other groups.

6. Visible toxic reactions as manifested by hematuria were present in 15.7 per cent of the rats receiving a combination of sodium bicarbonate and sulfadiazine as compared with 0 per cent in those in the other groups.

7. Blood sulfadiazine levels were essentially the same in the two groups of animals receiving the drug. The rats receiving the combination of sodium bicarbonate and sulfadiazine had a 15 per cent higher level of free sulfadiazine forty-eight and seventy-two hours after institution of therapy.

The pooled blood of ten rats was used for sulfonamide determinations in each of the two groups. At forty-eight and seventy-two hours, respectively, the free sulfadiazine levels were 10.8 mg. per cent and 12.0 mg. per cent in the rats receiving sulfadiazine and sodium bicarbonate, as compared with 9.5 mg. per cent and 10.8 mg. per cent in those receiving sulfadiazine alone.

### SURVIVAL PERIODS OF RATS WITH TYPE I PNEUMOCOCCAL INFECTIONS

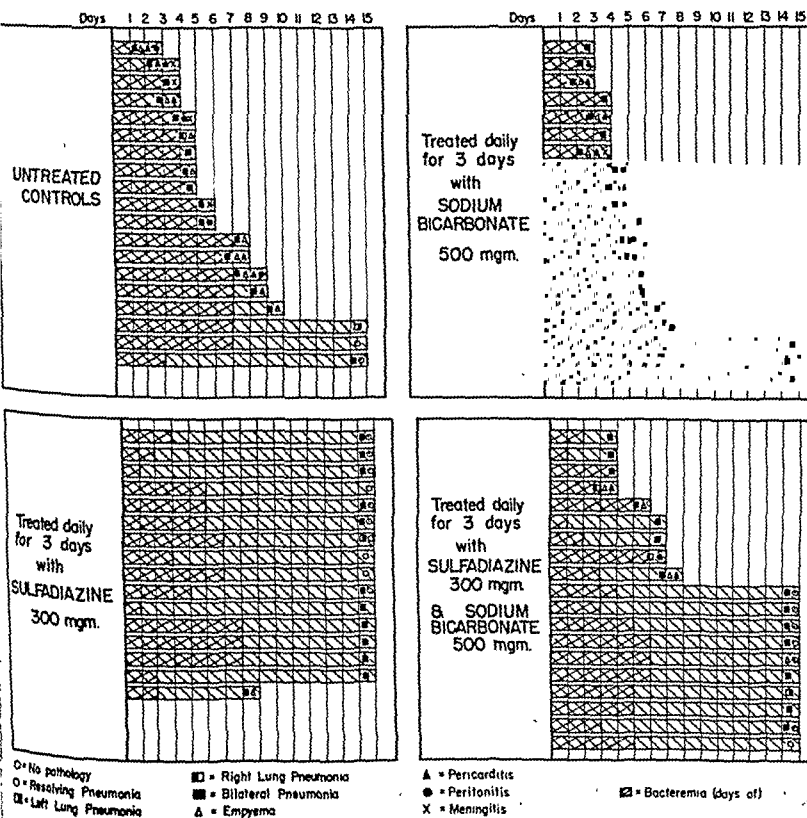


Fig. 3.



## CONCLUSIONS

This experiment has shown that in rats sodium bicarbonate exerts a deleterious action in pneumonia. This effect can be seen in the rats receiving only sodium bicarbonate but is more clearly seen in those receiving the combination of sulfadiazine and sodium bicarbonate. We have no experimental evidence, as yet, to offer as an explanation for this phenomenon.

We wish to express our appreciation to Dr. William Dock for his careful criticisms and suggestions in this study.

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## THE ACTION OF $\beta$ -DIMETHYLAMINOETHYL BENZILATE HYDROCHLORIDE, A SYNTHETIC ANTISPASMODIC

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IN THE development of new synthetic antispasmodics, esters of various organic acids and amino alcohols have outstanding possibilities, according to a recent review by Raymond.<sup>1</sup> Blicke and Kaplan<sup>2</sup> prepared a series of fifty-two such esters and found certain ones to possess local anesthetic and mydriatic actions. Furthermore, in this laboratory, tests of some of these esters have revealed antispasmodic activity. One compound in particular, the benzilic acid ester of dimethylaminoethanol,\* seems worthy of extensive study because of its high activity. The following report deals with an investigation of its pharmacologic properties.

### GENERAL PROPERTIES

The hydrochloride of dimethylaminoethyl benzilate is a white crystalline powder, soluble in water, and melts at from 183 to 185° C. When placed on the tongue, it is slightly bitter and produces a sensation of numbness. It is

From the Lilly Research Laboratories, Eli Lilly and Company.

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\*This substance, first prepared by Blicke, was kindly supplied to us by Mr. H. A. Shonle, of our organic chemical department.





*Action in Unanesthetized Dogs.*—Three dogs, each with a fistula of the stomach, were trained to lie quietly on a table while gastric motility was recorded by the balloon and water-manometer technique of Carlson.<sup>8</sup> Lehmann and

25MG HK141

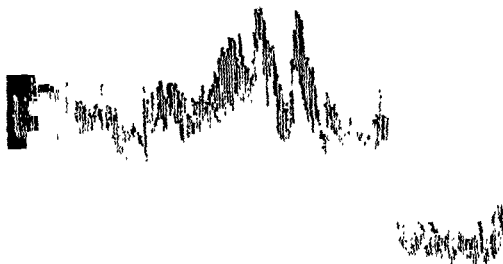


DOG NO 960

WT. 14.0KG

MINUTES

Fig. 3.—The inhibition by HK-141 of hunger contractions in a trained unanesthetized dog. For recording purposes, a balloon connected to a water manometer was introduced into the stomach via a fistula. The arrow indicates a dose of HK-141 administered through the fistula.



DOG NO. 204 WT. 10.0KG.



3MG HK141

MINUTES

Fig. 4.—Depression of the small intestines by HK-141. Intestinal movements were recorded by a water manometer connected to a small balloon in a Thiry fistula of a dog. HK-141 administered intravenously produced rapid decrease of tone but little or no effect on segmental movements.

Knoefel,<sup>7</sup> in their study of some related compounds, employed a similar procedure, except that they produced gastric hypermotility by insulin injections. In our experience, we found that it was necessary to starve the animals for twenty-four hours only before strong gastric contractions appeared. After starting the record, a hunger period was allowed to proceed until contractions were vigorous. In the middle of the period of hunger contractions, as judged by control experiments, an intravenous injection of from 2 to 5 mg. of HK-141 (total dose) produced prompt inhibition of the hunger contractions, usually of one-half hour's duration. The same effect occurred when the drug was introduced directly into the stomach through the fistula, although under these circumstances, there was a ten- to fifteen-minute latent period. By stomach, the effective dose was 2.5 to 10 mg. (total) and the duration of action about the same as that for the intravenous dose. These results were obtained from a total of eighteen experiments performed on these three dogs. In Fig. 3 is seen a record of the action of HK-141 on hunger of one of them.

The effect of the compound on intestinal motility was tested in five experiments on a dog with a Thiry fistula of the ileum. Means of recording were the same as for hunger; however, a smaller balloon was necessarily used. The intravenous injection of from 0.5 to 10 mg. produced a decrease of tonus of temporary duration. In Fig. 4 is shown a record of this effect. There apparently was little or no action on the segmental movements.

#### MYDRIATIC AND LOCAL ANESTHETIC PROPERTIES

Solutions of HK-141 in various concentrations were instilled into the conjunctival sac of seven albino rabbits. When the percentage was 0.05 or greater, these solutions produced dilatation of the pupil and inhibition of the light reflex similar to those produced by atropine sulfate. A 1.0 per cent solution similarly applied to the eyes of three rabbits caused anesthesia of the cornea of fifteen to twenty minutes' duration. No evidence of irritation was noted at any time. These results are in general agreement with those of Blicke and Kaplan.<sup>2</sup>

#### ACTION ON CIRCULATION AND RESPIRATION

In four cats anesthetized with sodium amytal, blood pressure and respiration were recorded in the usual manner. Intravenous doses of HK-141 of 1.0 mg. or less were generally without effect on either blood pressure or respiration. When anesthesia was light, doses of from 10 to 20 mg. caused a moderate stimulation of respiration and a blood pressure fall of from 20 to 80 mm. Hg of a few minutes' duration. Under deep anesthesia, injection of from 5 to 10 mg. resulted in a rise of from 30 to 70 mm. Hg with slight respiratory depression. Doses of from 20 to 40 mg. produced a marked fall of blood pressure and frequently were fatal. Respiratory changes probably were secondary to blood pressure variations, since the former appeared shortly after onset of the latter.

#### EFFECT ON SALIVARY SECRETION

In a dog anesthetized with seconal sodium, Wharton's duct was cannulated and connected to a drop recorder. In order to obtain a fairly rapid flow of saliva, pilocarpine was injected subcutaneously a few minutes beforehand. Intravenous administration of 0.1 mg. of HK-141 caused a prompt decrease in drop rate from 8 to 2 per minute, with recovery to the original rate in nine minutes. The same dose of atropine sulfate was somewhat more potent, causing depression of drop rate from 8 to 1 per minute and return to original in seventeen minutes.

## TOXICITY STUDIES

**Acute Toxicity in Mice and Rats.**—The median lethal dose  $\pm$  standard error was determined both by oral and intravenous administration to starved albino mice and rats. For comparison, similar determinations were made with syntropan given intravenously to mice and rats and with atropine sulfate administered by vein to mice. Computations were made according to the method of Bliss.<sup>6</sup> Results are shown in Table I. HK-141 was somewhat more toxic than either syntropan or atropine sulfate. However, the lethal oral dose was quite large for both rats and mice, which may indicate that this substance has a relatively low toxicity. Furthermore, HK-141 is much more active as an antispasmodic than syntropan, so the margin of safety of the former should be greater than that of the latter.

TABLE I. THE MEDIAN LETHAL DOSES (LD<sub>50</sub>'s) OF HK-141, SYNTROPAN, AND ATROPINE SULFATE IN MICE AND RATS

DRUG	ANIMAL	ROUTE OF ADMINISTRATION	NUMBER ANIMALS USED	LD <sub>50</sub> $\pm$ STANDARD ERROR (MG. PER KG.)
HK-141	Mouse	Intravenous	60	39.9 $\pm$ 2.2
		Oral	50	281.0 $\pm$ 15.1
	Rat	Intravenous	29	29.9 $\pm$ 1.9
		Oral	54	1035.0 $\pm$ 35.0
Syntropan	Mouse	Intravenous	35	51.1 $\pm$ 2.1
	Rat	Intravenous	26	42.8 $\pm$ 2.5
Atropine sulfate	Mouse	Intravenous	36	77.5 $\pm$ 3.5

**Feeding Experiments in Rats.**—Employing the method of Anderson, Henderson, and Chen,<sup>9</sup> young rats weighing about 80 grams were given a diet to which HK-141 had been added. Six groups of five rats each were used, each group receiving a different percentage of the drug. The percentages tested were 0.05, 0.1, 0.2, 0.3, 0.5, and 1.0. Food and drug intakes were recorded daily, while body weights were taken twice a week. The procedure was continued for twenty-eight days. At the end of this period, the rats on the highest percentage were subjected to necropsy. All rats survived the test period, but the 1.0 per cent diet definitely depressed growth. Weight gain was affected very little with concentrations as high as 0.5 per cent. The post-mortem examination of rats whose growth was depressed revealed no pathologic changes. It appeared that HK-141 was relatively well tolerated by rats even in large doses and for prolonged periods.

## CONCLUSIONS

The compound  $\beta$ -dimethylaminoethyl benzilate hydrochloride (HK-141) was studied by a number of pharmacologic procedures with the following results:

1. The antispasmodic action of HK-141 is both neurotropic and musculo-tropic as demonstrated on isolated small intestines. Its ability to antagonize parasympathetic stimulants approaches that of atropine sulfate, while its direct action on smooth muscle is far greater than atropine sulfate and even stronger than papaverine. In both respects, HK-141 is much more potent than syntropan.
2. Isolated uterine strips of a rabbit are also depressed by this substance.
3. HK-141 has an antispasmodic activity equal to atropine sulfate when given by mouth to anesthetized cats.
4. HK-141 in doses of from 2 to 5 mg. intravenously, or 2.5 to 10 mg. orally, inhibits hunger contractions of unanesthetized dogs. Intravenously, from 0.5 to

10 mg. of the compound depress the tone of small intestines of a Thiry fistula dog.

5. The local anesthetic and mydriatic properties of HK-141 have been confirmed in rabbits.

6. Antispasmodic doses of this substance usually have no effect on blood pressure or respiration of cats. Under light barbiturate anesthesia, larger doses cause a fall of blood pressure and respiratory stimulation, while the reverse occurs with deeply anesthetized animals.

7. HK-141 depresses salivary secretion of a dog but is less active than atropine sulfate in this respect.

8. The median lethal doses of HK-141 in rats and mice by intravenous injection are smaller than those of syntropan and atropine.

9. Rats fed the compound in quantities as great as 1.0 per cent of the diet for twenty-eight days show no pathologic changes. This amount depresses growth, but percentages of 0.5 or less have no appreciable effect on weight gain.

The authors are indebted to Mr. R. C. Anderson, Mr. Wallace C. Wood, and Miss Nila Maze for their invaluable assistance in these experiments.

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#### THE SIGNIFICANCE OF VERY LOW VALUES FOR BLOOD UREA NITROGEN

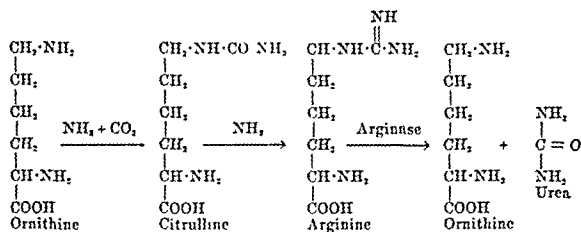
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ONE of the end products of protein metabolism in man is urea. This waste product is present in the blood stream and is excreted from the body mainly by the kidneys.

Formation of urea is limited almost entirely to the liver, as shown originally by Bollman, Mann, and Magath,<sup>1</sup> and confirmed by Krebs and Henseleit,<sup>2</sup> with the tissue slice technique. The latter workers proposed the modern con-

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cept of urea formation as due to the combination of carbon dioxide, ammonia from oxidative deamination of amino acids, and ornithine to give citrulline. Citrulline reacts with more ammonia to give arginine, which by the action of liver arginase is broken down to urea and ornithine for repetition of the cycle.



Since Krebs<sup>13</sup> review in 1936, Evans and Slotin<sup>4</sup> and Rittenberg and Waelsh<sup>5</sup> have shown, by means of isotope studies, that the carbon of urea is derived from carbon dioxide. Schönheimer and Rittenberg<sup>6</sup> have shown that the amidine group of arginine is derived from ammonia, with a similar technique.

Bach and Williamson<sup>7</sup> cast some doubt on the ornithine cycle as the sole mechanism for urea formation, but it is probably the major one.

Once formed, urea does not exchange amino groups with proteins or amino acids. Urinary ammonia is not derived from urea, as formerly taught, but from amino acids<sup>8</sup> by action of the amino acid oxidases of the kidney cortex.

The normal amount of urea in blood of man was determined by MacKay and MacKay<sup>9</sup> by 278 observations on 161 subjects. For males the range was 25.8 to 46.2 mg. per 100 c.c. and the mean 33.0. For females the range was lower, 11.0 to 39.0 mg., and the mean lower at 24.4. Blood samples were usually obtained in the afternoon and their values, for this reason, might be slightly higher than fasting values. They stated that "blood urea of lower than 10 mg. may be considered of questionable normality."

Osterberg and Keith<sup>10</sup> reported twenty-five cases in man of blood urea lower than 10 mg. per 100 c.c. for a four-year period. More females than males were present in the group. Every case was one of severe wasting disease, with either low protein intake or diuresis.

During a period of ten years, while about 80,000 blood urea determinations were done in our laboratory, we have noted many instances of very low blood urea, but we have been unable to explain all of them as Osterberg and Keith did.

#### METHOD

A modification of the van Slyke and Cullen aeration method was used, as follows:

Two or 3 c.c. of well-mixed oxalated blood, obtained from patients after from eight to twelve hours without food or drink, were pipetted into an eight-by-one-inch test tube. One cubic centimeter of a 16 per cent alcoholic extract of permittit-treated Jack bean meal and  $\frac{1}{2}$  c.c. of M/2 phosphate buffer at pH 7.0 were added and mixed with the blood and the tube incubated in a water bath at 50° C. for fifteen minutes. About 5 c.c. of distilled water and six drops of caprylic alcohol were added and the tube set up for vacuum aeration into 2 per cent boric acid solution with a methyl-red methylene-blue indicator. The stopper of the blood tube was removed, 5 c.c. of saturated solu-

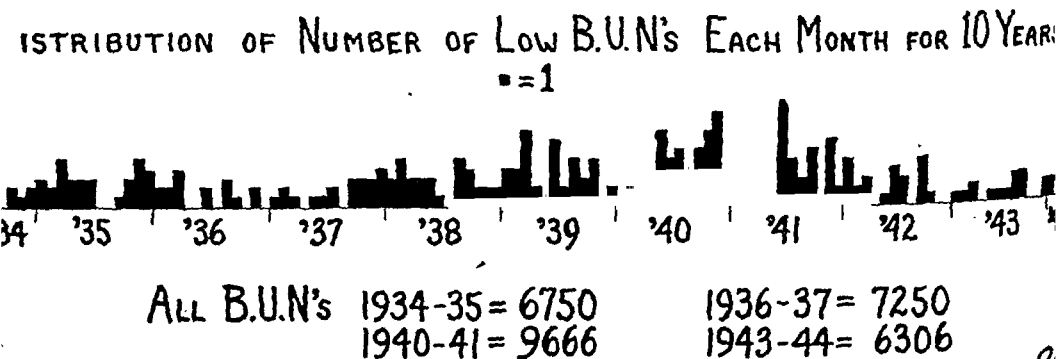


tion of sodium carbonate solution added, and the stopper replaced. Aeration was slow for five minutes, then at moderate speed for fifty-five minutes. After aeration the boric acid tube was titrated to its original end point (blue) with N/100 sulfuric acid. Final calculation was accomplished by multiplying the titer of N/100 sulfuric acid by 7 for 2 c.c. of blood or by  $14/3$  for 3 c.c. of blood. This gives a value for blood urea as urea nitrogen in mg. per 100 c.c. It can be converted to milligrams of urea by multiplying by 2.14, although it is our custom to express values as urea nitrogen.

All values to be reported were run in duplicate. Activity of the urease and accuracy of the method were checked with known urea solutions and by recovery of urea added to blood. All such checks proved the method was reliable and accurate.

#### DATA

From March 1, 1934, until March 1, 1944, among approximately 80,000 determinations of blood urea nitrogen, we have 315 determinations on 276 patients which were below our arbitrarily selected value of 5 mg. per 100 c.c. (equivalent to a blood urea of less than 10.7 mg. per 100 c.c.). In Fig. 1 is shown the incidence of these cases month by month throughout the period. Although there seems to be a higher incidence during 1940 and 1941, more urea nitrogen determinations were being done in our laboratory at that time. There is some correlation, but not a close one, between the total number of determinations of blood urea nitrogen (B.U.N.) and the incidence of very low values.



Hospital

Fig. 1.

Of the 276 cases, 210 were females and 66 were males. The sex distribution of patients in this general hospital is about half male and half female.

In Fig. 2 is shown the age distribution, ranging from 5 months to 79 years. The pattern resembles that of the age distribution of all patients admitted, and no correlation with age is warranted. The average age was 36.3 years.

The distribution of all very low values is shown in Fig. 3. Three instances of B.U.N. of less than 2.5 mg. per 100 c.c. were encountered in three patients. One was in a 28-year-old woman with acute gastroenteritis, who had values of 3.5, 3.0, 4.9, 2.1, 6.3, and 8 mg. per 100 c.c. on two admissions. Another was in a 23-year-old woman with chronic ulcerative colitis, who had values of 2.8, 2.1, 3.5, 5.6, 6, 6.3, 14, 5.6, and 5 during nine admissions. The third was in a 21-year-old woman, in the third month of pregnancy, admitted for ar-

thritis of one hand, who had only two determinations of 2.4 and 7. The first two had poor protein nutrition, while the third was well nourished. The low distribution of cases in the 3.6 to 4.0 mg. section is explained by the fact that most determinations were run on 2 c.c. samples of blood and the titer could be determined only to the nearest tenth of a cubic centimeter, so that values were 3.5 or 4.2, and seldom between these figures.

If we eliminate cases with B.U.N. over 4.6 mg., the limit set by Osterberg and Keith,<sup>9</sup> there are still 185 instances of very low values.

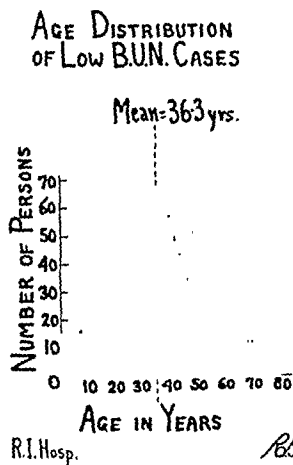


Fig. 2.

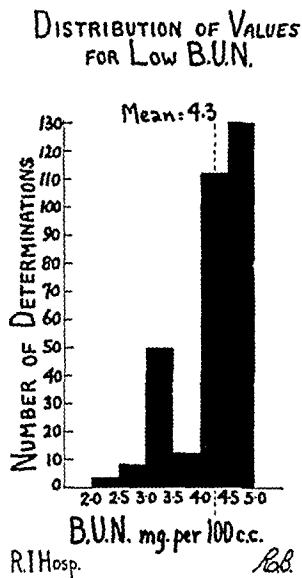


Fig. 3.

## DISCUSSION

In an attempt to explain why very low urea nitrogen may be present, the charts of 101 patients, on whom 126 determinations of B.U.N. below 5 mg. per 100 c.c. were obtained, were reviewed. The average age of those in the review group was 37.3 years, ranging from 5 months to 79 years, as for the whole group. There were seventy-two females and twenty-nine males. The mean B.U.N. was 4.1 mg. compared with 4.3 mg. for the whole group. The age distribution and incidence of values were similar to those for the whole group.

Because of Osterberg and Keith's findings, we tried to classify the reviewed cases as chronic wasting disease, chronic protein malnutrition, or active diuresis. Thirty-six were considered chronic wasting disease, with cancer, severe chronic asthma, chronic gastroenteritis, osteomyelitis, or prolonged fevers due to lung abscess, tuberculosis, thrombophlebitis, and endocarditis.

Most of these patients were in chronic protein malnutrition. Nine patients were considered to have protein deficiencies because of dietary habit or lack of intake of protein during postoperative convalescence. Eight patients, other than those mentioned, were in active diuresis for more than twenty-four

hours before the urea nitrogen determination. The remainder, forty-eight patients, were not in diuresis, had had good dietary protein intake, were in good physical condition, and had acute illnesses, or nonwasting chronic ones. As examples, a 52-year-old woman with chronic bronchitis, cor pulmonale, and mild pyelitis had a B.U.N. of 3.5 the day after admission; a 37-year-old woman, admitted for phenobarbital and bromide intoxication, had a B.U.N. of 3.5; a 32-year-old woman had B.U.N.'s of 3.5 and 4.2 on two different admissions for osteomyelitis of the fourth finger of the left hand caused by a human bite; a 59-year-old woman with mild diabetes mellitus and a mild chronic *Escherichia coli* cystitis had one B.U.N. of 3.5, two of 4.9, and twenty-two others ranging from 6 to 25 on three admissions, but showed low specific gravity and urine volume more than 1,500 c.c. previous to only one of the three very low values, a 4.9; a 60-year-old woman with a chronic gastric ulcer history admitted for obstruction had an aluminum hydroxide calculus removed from her stomach when her B.U.N. was 18, and one month later, just before discharge, she had a B.U.N. of 3.5; a 79-year-old woman with acute intestinal obstruction due to a gallstone had a B.U.N. of 27 on admission, one of 7 the day after operation, and one of 4.2 five days after operation, with daily urine volume never exceeding 1,200 c.c.; a 13-year-old boy admitted for acute osteomyelitis of the lower end of the tibia without fever had a B.U.N. of 4.2 twelve days after irrigation and drainage, two others of 6 before and after the 4.2; a 25-year-old man with edema of the legs and dyspnea had a B.U.N. of 4.2 when examined as an ambulatory patient at the cardiac clinic; a 50-year-old man admitted for dermatitis medicamentosa had a B.U.N. of 4.2; a 52-year-old man with second-degree burns of the right arm and shoulder had a B.U.N. of 3.5 on his second day in the hospital.

Dietary or therapeutic factors which might influence the urea nitrogen were looked for, but none were found. The functional capacities of the liver and of the kidney were evaluated in each case. No evidence of liver disease was obtained in 85 of the 101 reviewed cases. In fourteen, there was minimal evidence for liver dysfunction, and in two there was marked liver damage.

Evidence of marked liver damage was based on history of alcoholic intake and ascites, hematemesis, low blood cholesterol, low hippuric acid excretion, and the clinician's opinion. Most of the cases without evidence had only history and physical examination to rule out liver damage. A few had other tests which were negative. None had enlarged liver, jaundice, or history of jaundice, hematemesis, ascites, or poor appetite. One of the cases with marked liver damage also had normal and abnormally high urea nitrogen values. We have seen many other cases of marked liver damage with increased blood urea nitrogen, but only these two cases in the very low range.

The kidneys were presumed to be normal in eighty-three patients, were suspected of poor function in seventeen, and were markedly damaged in one. Osterberg and Keith<sup>9</sup> had one low urea in a patient who died later in uremia. Our patient had carcinoma of the cervix, grade III, bilateral pyelitis, and acute perinephritis. Her urea nitrogen rose from 15 to 36 before nephrostomy and abscess drainage, then fell to 5.6, rose to 17, and was 4.2 a few days before discharge with bilateral nephrostomy. She expired four months later at home.

Ten of the 101 patients died. Four were dead more than one day, but less than seven days, after the low urea nitrogen determination. Six others died more than seven days after the analysis. Thus the finding of a very low urea nitrogen is not a pre-mortem change. Schmidt and Carey<sup>11</sup> have noted an increase in urea nitrogen just prior to death.

We have no explanation for the higher incidence of females than males. The incidence is of the same order as that by Osterberg and Keith,<sup>9</sup> and MacKay and MacKay<sup>8</sup> found lower values in normal women than in normal men.

Although Osterberg and Keith<sup>9</sup> were able to explain their cases, we find about one-half of ours without explanation. We have many examples of chronic wasting diseases with poor protein intake, and with diuresis, yet with normal or even abnormally high urea nitrogen. There is no certainty that these factors will give very low urea nitrogen values. We are more inclined to believe that such instances are rare and are explained by temporary increases in kidney excretion of urea. Since urea is an important diuretic by itself, low blood urea would tend to decrease its diuretic action and lead to higher (normal) values later.

#### SUMMARY

Three hundred fifteen instances of blood urea nitrogen of less than 5 mg. per 100 c.c., in 276 hospital patients, were found in ten years among 80,000 analyses.

The very low figures for urea nitrogen were not found in any age group or correlated with diagnosis or therapy.

Very low figures were obtained three times as often in females as in males.

The blood urea nitrogen reaches a low of 2.1 mg. per 100 c.c. Values as low as 2.4 mg. per 100 c.c. may be obtained without evidence of diuresis, poor protein nutrition, or dysfunction of the liver.

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## LABORATORY METHODS

### A PROTECTIVE CABINET FOR INFECTIOUS DISEASE LABORATORIES

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SEVERAL procedures commonly employed in the investigations of certain of the infectious diseases are known to be dangerous. With the advent of the use of the embryonated egg for the growth of infectious agents it has seemed that laboratory infections have become more frequent. The membranes and other tissues of the egg at times are exceedingly rich in the infectious agent and often the procedures designed to disrupt these tissues tend to disperse finely divided droplets into the air. The Waring blender is an example of one such piece of laboratory equipment extensively used for the grinding of tissues that will on occasion leak and disperse infected material into the air.

Van den Ende§ has indicated the dangers inherent in intranasal inoculations of small animals. He has published a description of a box designed to protect the investigators engaged in such a procedure. The salient feature of this protective device consists of a constant draft produced by a gas burner with incineration of the contaminated air.

Using the basic principle of the box described by Van den Ende, we have designed a protective cabinet to house electrical equipment which may be a source of danger. The important detail of this cabinet is a guillotine type window which, when open more than  $\frac{1}{4}$  inch, throws an electrical limit switch, cutting off the power to the equipment inside.

*Description of the Protective Cabinet.*—The cabinet is constructed of wood and is made as nearly airtight as possible (Figs. A and B and Diagram 1). The floor is 30 by 30 inches, the sides are 24 inches in height, and the tapered top is approximately 12 inches in height and leads to the exhaust stack. The front consists of a snugly fitting vertical sliding window, counterbalanced by a sash weight and equipped with an electric limit switch to control power to the electrical outlets located inside the cabinet. There is mounted in the cabinet an ultraviolet light and an electric light for illumination, each of which is connected to an independent snap switch not in the circuit with the limit switch on the window. A baffle,  $8\frac{3}{4}$  inches square, of ordinary transite is inserted at the top of the box a short distance beneath the stack to interfere with the transmission of radiant heat from the stack.

The stack is 43 inches in overall height and 7 inches in diameter. It is composed of three sections: (A) a 6-inch length of galvanized sheet metal stovepipe which is fastened to the top of the cabinet; (B) the heater section of  $\frac{3}{32}$  inch black metal 18 inches long which contains the burner (Diagram 1,

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§Van den Ende, M.: An Apparatus for the Safe Inoculation of Animals With Dangerous Pathogens, J. Hyg. 43: 189, 1943.

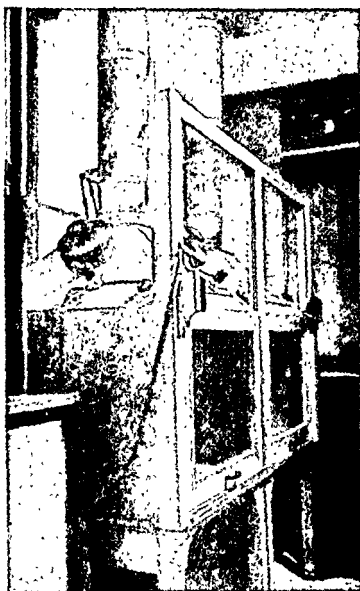


Fig. A.—Exterior view of two of the cabinets

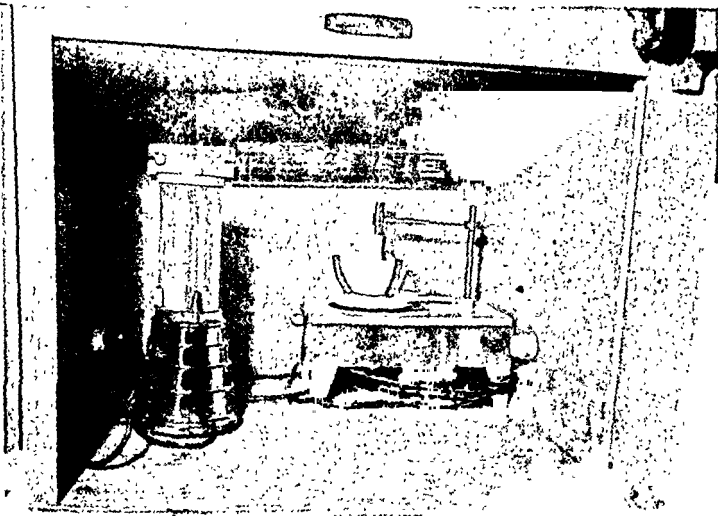


Fig. B.—Interior view of one of the cabinets with equipment.

## DETERMINATION OF AMINO NITROGEN OF BLOOD FILTRATES BY THE COPPER METHOD

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IN A PREVIOUS publication<sup>1</sup> which described the adaptation of the copper amino N method to the human urine, it was stated that due to sample size limitations the procedure could not be applied to blood filtrates. Experiments since then, however, have shown that by a suitable adjustment of reagent volumes and concentrations the copper technique can also be employed to advantage in the determination of human plasma or serum amino N. In these biologic fluids, as in the urine, it has been found that other N compounds, namely, creatine creatinine, uric acid, urea, and ammonia, do not participate in the copper reaction, so that it is possible to carry out the estimation without previous manipulation of the blood filtrate. This factor and the microquantitative characteristics of the reaction have been found to permit the accurate performance of this determination with a minimum expenditure of time and apparatus. The results obtained by the method compare favorably with those obtained with other techniques.

### EXPERIMENTAL

#### Reagents.—

*Cupric chloride*. 2 H<sub>2</sub>O: 27.3 Gm. are dissolved in 1 liter of water.

*Trisodium phosphate*: 64.5 Gm. are dissolved in 1 liter of water.

*Borate buffer*: To 57.21 Gm. of sodium borate in 1.5 liters of water contained in a 2 liter volumetric flask are added 100 c.c. of N HCl and the volume adjusted to the mark with water.

*Copper phosphate suspension*: 1 volume of cupric chloride solution is mixed with 2 volumes of trisodium phosphate solution and 2 volumes of borate buffer. This suspension keeps well in the refrigerator for about one week.

*Thymolphthalein indicator*: 0.25 Gm. of thymolphthalein (La Motte) is dissolved in 50 c.c. of 95 per cent ethanol and made to 100 c.c. with water.

*Sodium thiosulfate*: 49.6 Gm. (0.1 N) are dissolved in 200 c.c. of water and this solution is made to 2 liters after the addition of 20 c.c. of amyl alcohol. The amyl alcohol serves as a stabilizing agent. A 0.001 N solution is prepared and standardized as needed from the 0.1 N stock solution.

*Potassium iodate standard*: 356.7 mg. (0.01 N) of potassium iodate dried at 110° C. for one hour are dissolved in 1 liter of water; 1 c.c. samples of this solution are used to standardize the thiosulfate solution.

*Starch indicator*: 1 Gm. of Lintner solublized starch is dissolved in 100 c.c. of saturated NaCl solution by heating on the steam bath.

*Potassium iodide*: Made as needed by dissolving 1 Gm. of the salt per 1 c.c. of water.

*Trichloroacetic acid*: 10 per cent solution.

*Glacial acetic acid* and 1 N. NaOH.

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## PROCEDURE

**Collection of Sample.**—Two cubic centimeters of plasma or serum provide sufficient material for duplicate analyses. The plasma may be derived from blood collected over lithium oxalate or sodium citrate in the usual manner. The need for performing the estimation as soon after withdrawal of the blood as possible from the patient cannot be overemphasized. Hemolysis of the sample must also be avoided.

**Method.**—One cubic centimeter aliquots of plasma or serum are transferred by pipette to 15 c.c. conical centrifuge tubes containing 3 c.c. of 10 per cent trichloroacetic acid. After thorough mixing and standing for ten minutes, the tubes are centrifuged for five minutes at 3,000 r.p.m. The supernatant solutions are decanted through 2.5 cm. funnels containing paper filters (Whatman No. 5) into 100 by 13 mm. tubes and 2 c.c. aliquots are removed to graduated 15 c.c. conical centrifuge tubes. To each of these filtrates is added in succession a drop of thymolphthalein indicator, N. NaOH to the appearance of the blue color, 5 c.c. of copper phosphate suspension, and distilled water to the 10 c.c. mark. After being mixed by vigorous shaking, the reaction mixtures are allowed to stand for five minutes and then centrifuged for five minutes at 3,000 r.p.m. Five cubic centimeter aliquots of the supernatant fluid are removed from each tube to a 125 c.c. Erlenmeyer flask and acidified with 0.5 c.c. of glacial acetic acid. After the addition of 1 c.c. of KI solution and 3 drops of starch indicator, these samples are titrated with 0.001 N thiosulfate from a very fine-tipped 5 c.c. microburette. The end point is distinct and permanent.

**Calculations.**—Since each cubic centimeter of 0.001 N thiosulfate is equivalent to 0.028 mg. of amino N, the milligrams of amino N per 100 c.c. of plasma or serum are found from the following formula:

$$\text{c.c. of 0.001 N thiosulfate required for 5 c.c. of final filtrate} \times 0.028 \times 400$$

The agreement between duplicate determinations of the same sample is better than  $\pm 1$  per cent.

## RESULTS

Representative data from experiments designed to test the applicability of the method to blood filtrates by the recovery of amino N from added amino acids and non- $\alpha$ -amino nitrogenous compounds are given in Table I. For this investigation accurately weighed quantities of chemically pure compounds were dissolved in 10 per cent trichloroacetic acid and 3 c.c. aliquots of these solutions

TABLE I. RECOVERY OF AMINO N OF SUBSTANCES ADDED TO HUMAN PLASMA (1 c.c. OF POOLED HUMAN PLASMA, FOUND TO CONTAIN 0.0694 MG. OF AMINO N PER CUBIC CENTIMETER, USED FOR EACH TEST)

SUBSTANCES	NITROGEN OF COMPOUND ADDED		TOTAL AMINO N IN SAMPLE ANALYZED		RECOVERY OF ADDED AMINO N (PER CENT)
	TOTAL N (MG.)	AMINO N (MG.)	CALCULATED (MG.)	FOUND (MG.)	
Urea	1.40	0	0.0694	0.0689	99.3
Ammonium sulfate	0.93	0	0.0694	0.0708	101.5
Creatine	0.26	0	0.0694	0.0694	100.0
Creatinine	0.45	0	0.0694	0.0694	100.0
Uric acid	0.30	0	0.0694	0.0698	100.5
l (+) Arginine . HCl	1.420	0.355	0.4244	0.4260	100.9
dl-Lysine . HCl	0.698	0.348	0.4174	0.4180	100.2
dl-Alanine	0.972	0.972	1.0414	1.0250	98.5
dl-Methionine	0.503	0.563	0.6324	0.6230	98.6
l (-) Tryptophane	0.854	0.426	0.4954	0.4910	99.5
Amigen*	1.390	0.900	0.9694	0.9740	100.5

\*Kindly supplied by Mead Johnson & Co.; an enzymatic digest of casein containing 64.8 per cent of total N as free amino N.



were then employed as the protein precipitants for duplicate 1 c.c. portions of pooled plasma samples. Thus, it was found, as with the urine, that the non-amino substances do not participate in the copper reaction, whereas the amino acids uniformly yield values which are in quantitative accord with their amino N characteristics for the copper reaction. These findings further indicate that no appreciable loss of amino acids by adsorption is incurred from the trichloroacetic acid precipitation of the proteins.

TABLE II. AMINO NITROGEN CONTENT OF ADULT HUMAN BLOOD PLASMA AND SERUM\*  
(Mg. PER 100 C.C.)

NORMAL		ABNORMAL		
DONOR	AMINO N	PATIENT	DIAGNOSIS	AMINO N
A	7.64†	11	Cholecystitis	7.03
B	8.91	17	Nephrosis	7.33
I	5.78	19	Syphilis	7.51†
J	7.84	20	Syphilis	7.57
K	7.72	21	Diabetes	8.12
L	7.72	25	Diabetes	7.71†
M	7.94†	32	Pneumonia	7.92
N	7.48	40	Diabetes	7.36
O	8.28	42	Nephritis	8.96†
P	8.16†	43	Anemia	8.24
Q	8.62	46	Malaria	7.06
R	8.52			
S	8.40			
Average	7.94			7.71
Average deviation	±0.52			±0.36

\*Samples marked with dagger (†) were derived from sera; the remainder from plasmas.

The results of the analyses of normal and abnormal human blood plasmas and sera are given in Table II. The blood collections were all made from fasting subjects. A statistical treatment of the data from this limited series shows no significant differences between the plasmas and sera or normals and pathologic entities listed. In one instance, 1 c.c. aliquots of whole blood were analyzed and the amino N value found to be 16 per cent higher than that of the plasma derived from the same blood sample. In this connection, it is of interest to note that eight plasma and serum samples which showed varying degrees of hemolysis yielded an average amino N content of  $9.67 \pm 0.42$  mg. per cent. It is obvious from these observations that hemolysis of the sample must be avoided to obtain an accurate measurement of the plasma or serum amino N.

TABLE III. COMPARISON OF RESULTS OF AMINO N ANALYSES OF HUMAN PLASMA BY THE COPPER METHOD AND OTHER METHODS (Mg. PER 100 C.C. PLASMA)

INVESTIGATORS	METHOD	NUMBER OF CASES	AMINO N
Authors	Copper-amino	17	5.8- 8.9
Hamilton and Van Slyke <sup>2</sup>	Ninhydrin—CO <sub>2</sub>	13	3.4- 5.5
Cramer and Winnick <sup>3</sup>	Ninhydrin—CO <sub>2</sub>	20	2.3- 7.3
Kirk <sup>4</sup>	Gasometric	22	4.5- 8.1
Van Slyke and Kirk <sup>5</sup>	Colorimetric	9	4.8- 7.8
	Gasometric	9	7.5-15.7
	Acetone titration	9	7.2-15.3
	Formol titration	3	10.8-15.0

#### DISCUSSION

A summary of the data on amino N content of human plasma as reported by various investigators and the present authors is given in Table III. In general, the results of the various methods seem to agree as closely as could be expected considering the fact that the different methods do not determine exactly the same amount of amino N in each amino acid. The greatest differ-

ence is found in some of the high values secured by Van Slyke and Kirk<sup>5</sup> with the gasometric, acetone titration, and formol titration techniques, but these may have been due to some peculiarity of the unreported condition of the patients.

From the observations of others<sup>6</sup> and our own short series, the measurement of amino N level of the plasma appears to be of only limited diagnostic value. However, it is a valuable index in the study of the intermediary metabolism of amino acids and related physiologic experiments. In our experience the method described is ideally suited for investigations which demand numerous determinations, in that the procedure does not require the time-consuming preliminary treatment of the samples for the removal of urea, ammonia, or CO<sub>2</sub>, which is the case with the other available methods. An idea of the rapidity of operation of the copper amino N technique can be gained from the fact that a trained assistant can perform from ten to twelve analyses within two hours with an accuracy of better than 1 per cent.

#### SUMMARY

The copper amino N method has been successfully adapted for use with 1 c.c. of human serum or plasma. Analysis of the blood of thirteen normal subjects yielded an amino N value of  $7.94 \pm 0.52$  mg. per cent; and eleven abnormal cases,  $7.71 \pm 0.76$  mg. per cent. These data are compared with those obtained by other investigators employing the available procedures.

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## BOOK REVIEWS

**A Manual of Tropical Medicine.** By Colonel Thomas T. Mackie, M.C., A.U.S. Executive Officer, Tropical and Military Medicine; Chief, Division of Parasitology, Army Medical School; Major George W. Hunter, III, Sn.C., A.U.S., Division of Parasitology, Army Medical School; and Captain C. Brooke Worth, M.C., A.U.S., Division of Parasitology, Army Medical School. W. B. Saunders Company, Philadelphia, Pa. Price \$6.00. Cloth with 727 pages and 287 illustrations.

The authors have succeeded in condensing salient material and much information of practical value into a concise manual of tropical medicine. The material is presented briefly yet in sufficient detail to be completely understandable. The illustrations are particularly good. The charts depicting the epidemiology of various diseases are of considerable interest and serve to emphasize important features in the control of disease. Therapeutic measures are covered adequately.

Section X, under the heading Medically Important Arthropods, stresses the tremendous importance of the arthropod in world disease, 142 pages being devoted to entomology. Unnecessary biological details are omitted, yet the subject is well covered, showing clearly the role of the arthropod in disease processes. Table 49, *Human Disease Transmitted by Arthropods*, is an excellent summary of present-day knowledge on the subject. The entomological illustrations are good and should be of help to the novice who requires a working knowledge of this difficult and vast field.

The importance of laboratory procedures in the diagnosis of tropical disease is obvious. The authors have presented in Section XI, *Laboratory Diagnostic Methods*, a concise and clearly written outline of useful laboratory information. This portion of the manual should be especially helpful to the general practicing clinician who may not have adequate laboratory facilities at his disposal.

For military and civilian physicians *A Manual of Tropical Medicine* will serve as a useful and reliable guide through the welter of subjects included under the heading Tropical Medicine. For the medical student, the simple, clear, and concise presentation of material will be of assistance in the perusal of more detailed volumes on tropical medicine.

R. J. B.

**Penicillin Therapy Including Tyrothricin and Other Antibiotic Therapy.** By John A. Kolmer, M.S., M.D., Dr. P.H., Sc.D., LL.D., L.H.D., F.A.C.P. Professor of Medicine in the School of Medicine and the School of Dentistry, Temple University; Director of the Research Institute of Cutaneous Medicine; Formerly Professor of Pathology and Bacteriology, Graduate School of Medicine, University of Pennsylvania. D. Appleton-Century Company, Inc., 1945, New York, N. Y. Price \$5.00. Cloth with 302 pages.

The literature on penicillin has become extensive, as is always the case with any strikingly successful clinical procedure or agent. At the present time penicillin may be obtained by medical practitioners generally, so that the need for correlating and summarizing the knowledge about it for practical purposes is evident. In this book Dr. Kolmer has prepared an extensive review of the literature with references to most of the original work and has made a particular effort to give the details needed for clinical use of the drug. It seems to this reviewer that this is worth while and important but that he has gone too far in two respects. In his desire to include all possible details, he has given many that are unnecessary, such as a full-page illustration of a nurse injecting fluid into a vial with a syringe. Also, the numerous possible applications of penicillin therapy make a very large number of variations in dosage and methods of administration and there are often minor differences of opinion on these matters in individual situations. Dr. Kolmer has covered these points in a detailed and encyclopedic manner and I think has not emphasized sufficiently that the intelligent use of the drug must depend primarily on a knowledge of the fundamental principles of action and pathogenesis rather than on the exact procedure used by someone else in the same disease. A few important references have been omitted from the book, but on the whole it is valuable and may be recommended.

C. G. HARFORD.

**Penicillin and Other Antibiotic Agents.** By *Wallace E. Herrell, M.D., M.S., F.A.C.P.* Assistant Professor of Medicine, The Mayo Foundation, University of Minnesota; Consultant in Medicine, Mayo Clinic, Rochester, Minn. W. B. Saunders Co., Philadelphia, Pa. Price \$5.00. Cloth with 348 pages and 45 illustrations.

Perhaps no therapeutic agent was more critically evaluated in such a short period of time than was penicillin. This does not apply to the other antibiotics with the possible exception of tyrothricin. Intense interest has been manifested by the medical profession in the use of these agents, especially penicillin. In view of this, Dr. Herrell has attempted to crystallize the experimental and clinical studies carried out with penicillin and other agents. This is an aspiration of considerable magnitude.

The monograph is divided into four parts. Part I is an exposition of the well-known history of penicillin; its properties; pharmacology; and assay methods. In Parts II and III are detailed the clinical use of penicillin and its toxicity. In Part IV are discussed other antibiotic agents such as tyrothricin, streptothricin, and streptomycin. Chapters within each of these main sections are followed by references to the literature on the subject.

The text is well written and edited. It is not surprising that a definitive statement is not made concerning many aspects of the subject, such as the chemistry of penicillin and its mode of action, and the clinical use of streptomycin. Likewise, after reading the clinical sections, the physician will be left in a quandary as to the value of penicillin in such diseases as scarlet fever and peritonitis. Nevertheless, most of the information available has been included in this work. The section on tyrothricin is particularly well done.

The publishers are to be commended for the excellency of the print and reproduction of illustrations.

W. W. S.

**Microbial Antagonisms and Antibiotic Substances.** By *Selman A. Waksman, Professor of Microbiology, Rutgers University; Microbiologist, New Jersey Agricultural Experiment Station, New Brunswick, New Jersey.* The Commonwealth Fund, New York, N. Y. Price \$3.75. Cloth with 350 pages.

The recent discovery of the therapeutic value of certain antibiotic substances has resulted in a great wave of interest in the material dealt with in this book. As the title indicates, it is more than a treatise on antibiotic agents. Doctor Waksman has long been a leading figure in the field of soil microbiology, and since the soil is the source of most organisms known to produce antibiotic substances, it is not mere chance that he is now a leader in this new field. The book opens with a general discussion of soil and water as habitats of microorganisms. This discussion, dealing with the interrelationships between organisms in the soil, the physical and chemical nature of the medium, the fate of pathogens on introduction into the soil, pathogens of soil origin, etc., should prove very interesting and instructive to most bacteriologists and medical men, as well as other biologists who have been trained to think in terms of pure cultures and single infections instead of organisms in their natural habitats. Special attention is given to the subject of plant and animal wastes as related to soil populations. In bringing the reader closer to the major thesis of the book, such phenomena as synergism, symbiosis, parasitism, and antibiosis are discussed.

The fourth chapter deals with the more practical problems of isolation and cultivation of antagonistic organisms and the measurement of antibiotic action. The next several chapters are devoted to bacteria, actinomycetes, fungi, and animal forms as antagonists. In completing this aspect of the subject, a short chapter is devoted to the antagonistic relationships between bacteria and viruses and among viruses themselves.

The second half of the volume deals with more familiar subjects such as the chemical nature of antibiotic substances, the mechanism of antibiotic action, and disease control. In the final chapter Doctor Waksman speculates a bit on the future. No definite promises are made, but he leaves no doubt in the reader's mind that this is a field of science in which much may be expected. Already the problems have far outgrown the limits of microbiology, and as time goes on, the chemist, the chemotherapist, the physiologist, and other specialists will make substantial contributions in the development of this field.

This book is exceptionally well documented, having a bibliography of more than 1,000 references. Two extensive indexes are provided; one lists the microorganisms mentioned and the other covers the general subject matter. The work is very readable and should prove of much interest and value to all students of biology and medicine, and particularly to workers interested in the special problems of antibiosis.

J. ARTHUR HERRICK.

**Handbook of Practical Bacteriology.** By *T. J. Mackie*, C.B.E., M.D., D.P.H. Professor of Bacteriology, University of Edinburgh; Honorary Bacteriologist to the Royal Infirmary, Edinburgh; Director of Bacteriological Service, City of Edinburgh; Regional Director S.E. Scotland, Emergency Bacteriological Service; and *J. M. McCartney*, M.D., D.Sc., Director of Research and Pathological Service, London County Council; Major, R.A.M.C.; formerly Lecturer in Bacteriology, University of Edinburgh; Fellow of the Rockefeller Institute for Medical Research, N. Y. Seventh Edition. Williams & Wilkins Company, Baltimore Md. Price \$5.00. Cloth with 720 pages.

**Diagnostic Procedures and Reagents.** Second Edition. Edited by the American Public Health Association, New York, N. Y. Price \$4.00. Cloth with 549 pages.

**The Male Hormone.** By *Paul DeKruif*. Harcourt, Brace and Company, New York, N. Y. Price \$2.50. Cloth with 243 pages.

## PENICILLIN SENSITIVITY OF STRAINS OF SIX COMMON PATHOGENS AND OF HEMOPHILUS HEMOLYTICUS

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**S**UCCESSFUL penicillin therapy depends mainly on two factors: the susceptibility of the infecting bacteria and the availability of an adequate concentration of the effective agent to act upon the organisms. In general, the most gratifying clinical results have been obtained in those diseases in which the causative organism is susceptible in vitro to concentrations of penicillin which can readily be maintained in the patient. A knowledge of the relative susceptibility of different strains of the common pathogenic organisms, as well as acquaintance with the general range of concentrations to be expected from the use of various doses, intervals, and routes of administration, is, therefore, essential to the intelligent management of penicillin therapy.

In this paper are presented the results of tests for sensitivity to commercial penicillin carried out on a number of strains of common bacteria. Other quantitative aspects of penicillin therapy will be dealt with in separate communications.

### MATERIALS AND METHODS

The penicillin sensitivity of the different strains was tested in suitable media by the serial dilution method of Rammelkamp and Maxon<sup>1</sup> modified by the use of a final volume of 1.0 c.c. A strain of hemolytic streptococcus, No. 98, obtained from Dr. C. S. Keefer was used as a standard reference strain throughout this study. Broth cultures containing 1.0 per cent horse blood and seeded with approximately 10,000 of the standard streptococci per cubic centimeter were consistently sterilized by 0.0078 units of commercial penicillin in eighteen hours. Penicillin solutions were prepared at intervals from pools of five vials of commercial penicillin in sterile physiologic saline and stored at 5° C. End points were read as the smallest amounts of penicillin in which there was no evidence of growth at eighteen hours and which yielded no growth in transplants on blood agar after another twenty-four hours of incubation. The crudeness of such a biologic test and its broad limits of error are well recognized.

Most of the strains included in this report were obtained during the course of treatment of various infections on the adult medical wards or during clinical trials of various forms of treatment in cases of acute gonorrhea in the out-patient genitourinary clinic.\* Several strains of meningococcus isolated from patients and carriers were obtained through the courtesy of Major Emanuel B. Schoenbach.

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Part of the penicillin used in this study was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

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## RESULTS

The susceptibility of a number of strains of each of seven different organisms is shown in Fig. 1 and is indicated both in terms of Oxford units of commercial penicillin and in comparison with streptococcus 98. The variability in the resistance of these organisms and among the different strains of the same organism is at once apparent and has already been noted by Fleming<sup>2</sup> and others.<sup>3-9</sup>

	GONOCOCCUS	BETA STREPTOCOCCUS	PNEUMOCOCCUS	ALPHA STREPTOCOCCUS	HEMOPHILUS HEMOLYTICUS	MENINGOCOCCUS	STAPHYLOCOCCUS AUREUS	
256							5	2.0
64					1	1	1	0.5
32					2	5		0.25
16				1	3	9	1	0.125
8			2	1	7	9	9	0.063
4			26	13	8	27	14	0.031
2		1	50	8		3	6	0.016
1	1	12	10	1				0.008
1/2	18	1						0.004
1/4	5							0.002
	NUMBER OF STRAINS							OXFORD UNITS PER C.C.

Fig. 1.—Penicillin sensitivity of 261 strains of seven different bacteria compared with the reference strain of hemolytic streptococcus, No. 98.

*Gonococcus*.—Gonococcus was the most sensitive of the organisms studied. Almost all of the strains were two or four times as sensitive as the reference strain of streptococcus 98. They were all obtained from cultures of cervical or urethral discharge of patients with acute gonorrhea. The majority were sulfonamide resistant, but there was no correlation between the susceptibility to sulfonamides and to penicillin. Similar findings have been reported by others,<sup>10, 11</sup> some of whom have succeeded in increasing the resistance of these organisms in vitro,<sup>12</sup> but naturally resistant strains have not been encountered.

*Hemolytic Streptococcus*.—Strains of Group A beta hemolytic streptococci were most uniform in their susceptibility to penicillin, almost all of them resembling streptococcus 98 in that respect. The strains listed are chiefly from pharyngeal and blood cultures. A considerably larger number of strains from patients with scarlet fever have also been tested and showed similar sensitivity to penicillin. At least twelve distinct types were represented, but there was no relation between type and penicillin sensitivity among these Group A strains.

Naturally resistant group A strains have not been encountered, but resistance has been produced in vitro<sup>13</sup> and has been found occasionally to increase during therapy.<sup>8</sup> Other Lancefield groups were not studied, but Watson<sup>14</sup> showed that only strains of Group D, *Streptococcus fecalis*, are insensitive, a finding which has been confirmed with respect to strains isolated from urinary tract infections.<sup>15</sup>

*Pneumococcus*.—The eighty-eight strains of pneumococci represented twenty-four specific types and were isolated chiefly from sputum and blood of patients with pneumonia before treatment. They varied in sensitivity from that equal to the control streptococcus 98 to being eight times more resistant. There was no correlation between the type and the sensitivity of the different strains. Naturally resistant pneumococci have not been encountered. Resistance of pneumococci to penicillin has been increased experimentally without altering their response to sulfonamides<sup>13, 16</sup> or their virulence.<sup>13</sup> Conversely, the development of sulfonamide fastness was found to have no effect on the response to penicillin.<sup>17</sup>

*Alpha Streptococcus*.—Most of the twenty-four strains of alpha streptococcus listed in Fig. 1 were isolated from the blood of patients with subacute bacterial endocarditis, but a few were obtained in pure culture from infected body fluids or as the predominant organism from nasopharyngeal cultures. Most of them were two or four times more resistant than strain 98. The least sensitive strain was sixteen times more resistant than the control, and the most susceptible one was the same as the control strain. Strains isolated after a course of penicillin therapy in patients with subacute bacterial endocarditis showed differences in cultural characteristics as compared with the original strains, but in one instance a later strain was found to be four times more sensitive than an earlier one.<sup>18</sup> Greater strain variability and the finding of naturally resistant strains have been reported.<sup>2, 6, 19, 20</sup>

*Meningococcus*.—The fifty-four strains of meningococcus tested were isolated from the blood or spinal fluid of patients with meningitis or from pharyngeal cultures of patients or carriers. They showed a wide range of sensitivity to penicillin, varying from two to sixty-four times more resistant than streptococcus 98. At least two of the more resistant strains were original strains isolated from patients who showed a poor response to treatment with intramuscular and intrathecal penicillin and subsequently responded favorably to sulfonamides.<sup>21</sup> Most of the strains were also tested for sensitivity to sulfonamides and found to be highly susceptible. Serologically, thirty-nine of the strains were classified in Group I, four in Group II, three in Group II<sub>a</sub>, and eight could not be classified. There was no correlation between the serologic type and the sensitivity to penicillin.

*Staphylococcus aureus*.—The thirty-six strains of *Staph. aureus* tested were mostly hemolytic and coagulase-positive strains isolated from the blood or exudates of patients with pneumonia, empyema, arthritis, or acute osteomyelitis. The individual strains showed the widest variation in susceptibility to penicillin, ranging from 2 to 264 times more resistant than the reference strain 98. For at least six of the strains the inhibiting concentrations of penicillin were greater than those which can be readily maintained in the serum during therapy. The occurrence of naturally resistant pathogenic strains of *Staph. aureus* and the development of increased resistance of susceptible strains in vitro or during penicillin therapy have been reported.<sup>1, 4, 7, 9, 23, 22-27</sup> Nine of the strains were nonhemolytic and five were coagulase negative, but there was no correlation between penicillin sensitivity and the hemolytic or coagulase activity.



*Hemophilus hemolyticus*.—Interest in the susceptibility to penicillin of this apparently nonpathogenic organism was aroused during the course of studies on the effect of a penicillin spray in patients with acute pharyngitis. In some cases in which almost pure cultures of these pleomorphic, gram-negative bacilli were grown from the throat, they were found to disappear rapidly from pharyngeal cultures taken after treatment was begun. The twenty-one strains tested were isolated from throat cultures of patients with acute pharyngitis or from sputum cultures from patients with pneumonia. The range of sensitivity of these strains was found to be similar to that of the great majority of strains of meningococcus and of *Staph. aureus*. They ranged from four to sixty-four times more resistant than streptococcus 98, most of them being only four or eight times more resistant. The only other gram-negative bacillus which has been shown to have a similar sensitivity to penicillin is *Hemophilus ducreyi*.<sup>28</sup> Strains of *Hemophilus influenzae* and *Hemophilus pertussis* have all proved to be highly resistant.<sup>2, 28</sup>

#### DISCUSSION

The results presented confirm previous reports on the wide variations in susceptibility among organisms generally considered to be sensitive to penicillin as well as among different strains of the same organism. A general acquaintance with the relative sensitivity of the common pathogenic organisms may be of considerable help in the intelligent management of penicillin therapy.<sup>29, 30</sup>

Almost all of the strains of organisms dealt with in this paper have been isolated in relation to penicillin therapy. Except for *H. hemolyticus*, which is included here only because of its peculiar interest as a penicillin-sensitive gram-negative bacillus, these organisms are representative of the great majority of susceptible pathogens encountered in general medical practice. Among these common pathogens only some of the strains of *Staph. aureus* were sufficiently resistant to suggest that they might not yield to systemic therapy as now generally used. Other staphylococcus strains and some strains of meningococcus seem to be sufficiently resistant to require more than average dosage in order to maintain bacteriostatic levels. Other reports suggest that certain strains of alpha or gamma streptococci from patients with subacute bacterial endocarditis likewise require relatively high concentrations of penicillin for the optimum effect.

Meningococci, fortunately, are quite uniformly sensitive to sulfonamides. Penicillin-resistant staphylococci and streptococci, on the other hand, still pose a challenging therapeutic problem because the focal lesions which they produce may be inaccessible for topical use of the antibiotic in sufficient concentration. In addition, these organisms are relatively resistant to sulfonamides as well.

The contrast between the gonococcus and the meningococcus is of some interest. Both are usually considered to be sensitive to penicillin and to sulfonamides. The gonococcus, however, appears to be considerably more susceptible to penicillin, while the meningococcus seems to be much more sensitive to sulfonamides. Sulfonamide resistance is apparently readily induced during treatment of gonococcal infections. At any rate, sulfonamide-resistant strains of gonococci are being encountered with increasing frequency, whereas such resistance has not been encountered among meningococci. The reverse, however, seems to be true with respect to penicillin—relatively resistant strains are encountered among meningococci but not among gonococci.

The exact nature of penicillin resistance is still a matter for speculation. The majority, though not all, of the naturally resistant organisms have been

found to contain or to produce a penicillin-inactivating enzyme.<sup>24, 31-33</sup> Development of penicillin resistance, however, was found not to be associated with the acquisition by the bacterium of the ability to produce such a penicillinase.<sup>24</sup> There is some evidence that the development of resistance by bacteria exposed to proper concentrations of penicillin may be a process of selection by which susceptible individuals are gradually eliminated as the concentration of the antibiotic is increased. This leaves the more resistant individuals which then reproduce further generations of resistant individuals.<sup>29</sup>

## SUMMARY

Tests for sensitivity to commercial penicillin in vitro have been carried out on 240 pathogenic strains of gonococcus, beta and alpha streptococcus, pneumococcus, meningococcus, and staphylococcus and also on twenty-one respiratory strains of *H. hemolyticus*. There were wide differences in sensitivity among these organisms and among different strains of the same organism.

The strains of gonococcus and of Group A hemolytic streptococcus were the most sensitive to penicillin and showed the greatest uniformity in that respect. The strains of staphylococcus and meningococcus showed the widest range of sensitivity. Most of the *H. hemolyticus* strains reacted like the majority of staphylococci and meningococci. The sensitivity of strains of pneumococcus and alpha streptococcus was intermediate between that of beta streptococci and meningococci.

Resistant strains, which were 256 times more resistant than the reference strain of hemolytic streptococcus, were encountered only among the staphylococci. Relatively insensitive strains, which were from sixteen to sixty-four times more resistant than the reference strain, were found among the staphylococci, meningococci, *H. hemolyticus*, and alpha streptococci.

Among the Group A streptococci, pneumococci, and meningococci there was no correlation between the serologic types and penicillin sensitivity. Among the pathogenic staphylococci there was no apparent relation between penicillin sensitivity and the hemolytic or coagulase properties of the different strains.

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## THE SUPERIORITY OF THE CONTINUOUS INTRAVENOUS DRIP FOR THE MAINTENANCE OF EFFECTUAL SERUM LEVELS OF PENICILLIN: COMPARATIVE STUDIES WITH PARTICULAR REFERENCE TO FRACTIONAL AND CONTINUOUS INTRAMUSCULAR ADMINISTRATION

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THE treatment of infections with penicillin has been largely empiric, and dosage schedules generally have been evolved by trial and error. Great stress has been placed upon the sensitivities to penicillin of various organisms which have been classified as sensitive or insensitive. Few assays have been made upon body fluids in order to determine whether effective penicillin levels have been obtained and whether they were being maintained.

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The treatment of certain diseases such as gonorrhea is relatively simple because of the extreme sensitivity of the infecting organism to small doses of penicillin. The treatment of diseases such as thrombo-ulcerative endocarditis due to alpha or gamma streptococci is fraught with difficulties because of the great variability in the sensitivity of the infecting organisms and the need for prolonged adequate dosage. It is highly desirable to be able to predict, if only approximately, what blood penicillin levels may be obtained and how long they will be sustained with a given technique of administration. Studies of this type have been limited. Rammelkamp and Keefer<sup>1</sup> have shown that the intravenous injection of a single dose of penicillin resulted in a very rapid rise and a rapid fall of the blood level so that at the end of two hours no detectable amounts remained. Absorption was somewhat slower and blood levels were better maintained following intramuscular administration. The subcutaneous administration was followed by irregular absorption and low blood levels. Cooke and Goldring<sup>2</sup> have demonstrated similar findings in children treated with penicillin.

Rantz and Kirby<sup>3</sup> have reported on the absorption and excretion of penicillin following continuous intravenous and subcutaneous administration. They find an approximately linear relationship between plasma penicillin levels and the rate of intravenous penicillin infusion when the material is given at a constant rate of flow. The continuous subcutaneous method was associated with plasma levels much lower than those following the intravenous method. The dosage schedules used did not exceed 480,000 units per day; clearance studies indicated that the maximal tubular excretory capacity of the kidneys had not been reached with this amount.

#### METHODS AND MATERIALS

**Penicillin:** Penicillin was originally supplied as the sodium salt, powdered, dehydrated, and in vacuo, in bottles each containing 100,000 units. Appropriate amounts were dissolved in sterile, pyrogen-free isotonic saline or Ringer's solution. With massive dosages, up to 10,000,000 units daily, the solution of so much dry powder proved arduous and entailed considerable loss so that sterile solutions\* of sodium penicillin were especially prepared in a concentration of 100,000 units per cubic centimeter.

**Penicillin Assay:** Serum and urine were titrated routinely for penicillin content by the microbiologic method of Rosenblatt, Altire-Werber, Kashdan, and Loewe.<sup>4</sup>

**Blood:** Blood was collected aseptically by syringe and transferred immediately to a sterile test tube. This was allowed to clot in the refrigerator and the separated serum was assayed for its penicillin content.

**Administration:** When given by continuous intravenous drip,<sup>5-8</sup> suitable amounts of penicillin were dissolved in 1,000 c.c. of either isotonic saline or Ringer's solution. This was allocated over a twenty-four hour period. With intramuscular dosage, the appropriate amount of penicillin was dissolved in 2 c.c. of isotonic saline or 1 per cent novocain solution. The anesthetic vehicle was used if the subjects complained excessively of local pain. It was previously determined that 1 per cent novocain solution had no antibiotic influence.

**Subjects:** The subjects were all adult patients under treatment for subacute bacterial endocarditis.<sup>9-11</sup> The sexes were approximately equally repre-

\*We are extremely indebted to Mr. John L. Smith, of the Charles Pfizer Company, for his keen interest, valuable suggestions, and constant cooperation. Through him we were enabled to obtain the lavish supplies of penicillin utilized in all our experimental studies. The especially prepared solution of penicillin proved to be convenient, time saving, and stable at refrigerator temperature over a period of at least two weeks.

sented. No attempt was made to correlate body weight, habitus, or temperature with dosage or blood levels.

*Serum Penicillin Concentrations Following Fractional Intramuscular Dosage.*—The most popular mode of penicillin administration is fractional intramuscular injection. In Table I is a compilation of average blood levels obtained in several hundred determinations done on approximately 100 different subjects. It must be emphasized that these are average curves for given dosage schedules. Individual determinations on the same or different subjects may vary from the norm by as much as 25 to 100 per cent.

TABLE I. PENICILLIN SERUM LEVELS FOLLOWING FRACTIONAL INTRAMUSCULAR ADMINISTRATION

PENICILLIN DOSAGE (OXFORD UNITS)		SERUM TITER (OXFORD UNITS PER C.C.)			
INDIVIDUAL DOSE	EQUIVALENT DAILY DOSE (EVERY 2 HR.)	30 MINUTES	60 MINUTES	90 MINUTES	120 MINUTES
10,000	120,000	0.15	0.06	—	—
16,666	200,000	0.15	0.06	0.01	—
20,000	240,000	0.2	0.09	0.035	0.01
25,000	300,000	0.35	0.2	0.063	0.03
40,000	480,000	0.5	0.33	0.22	0.09
41,666	500,000	0.7	0.45	0.25	0.15
50,000	600,000	1.0	0.6	0.3	0.2
83,333	1,000,000	1.5	0.75	0.4	0.25
100,000	1,200,000	1.7	0.8	0.625	0.3

*Results:* Maximum serum levels are obtained within thirty minutes. At the end of one hour, levels fall sharply to approximate 50 per cent of the thirty-minute figure. Thereafter the drop is less precipitate, but at the end of an hour, significant amounts of detectable penicillin are not present in the serum except with high dosage.

*Comment:* When the sensitivity of an infecting organism is known, it is a simple matter to choose the proper dosage schedule. For example, most strains of beta hemolytic streptococci are highly susceptible to penicillin and are inhibited in vitro in concentrations of from 0.008 to 0.015 unit per cubic centimeter. Dosages of penicillin between 20,000 and 25,000 units administered every two hours should satisfactorily inhibit this type of organism.

The relatively resistant organisms such as the alpha and gamma streptococci, usually found in thrombo-ulcerative endocarditis, present a more difficult problem. Many strains in these groups have a penicillin sensitivity of from 0.15 to 0.6 unit per cubic centimeter or higher. With intramuscular dosage the ninety-minute or two-hour serum penicillin level is almost always below the in vitro sensitivity of the organism. Thus, in effect, therapy may be ineffectual for a significant portion of the time between injections. Situations of this type are hazardous, for there is evidence to prove that inadequate dosage may result in treatment failure due to acquired increased resistance<sup>12</sup> of organisms to the action of penicillin.

Many patients do not tolerate fractional intramuscular injections despite the fact that different sites are chosen for injection. Local pain may be alleviated by using a solution of novocain as a vehicle, but this is not suitable for long courses of therapy. Furthermore, the necessity for frequently repeated injections interferes with the rest and morale of the patient.

Briefly, in the treatment of disease due to bacteria which are highly susceptible to the action of penicillin and where the span of therapy is relatively short (for example, gonorrhea, pneumonia), the fractional intramuscular method of penicillin administration can be recommended.

*Serum Penicillin Concentrations Following Continuous Intramuscular Drip.*

—The administration of penicillin by continuous intramuscular infusion has been recommended by Harris<sup>13</sup> and by Dawson and Hunter.<sup>14</sup> The latter authors report that this method of administration appears to be the procedure of choice for patients receiving penicillin for prolonged periods. They state that comparative studies usually show that higher blood levels are obtained by continuous intramuscular drip than by continuous intravenous drip. They consider intramuscular drip technically simpler and better tolerated by the patient.

*Experimental:* Comparative levels were determined in a number of patients. The daily dosage of penicillin varied from 500,000 to 5,000,000 Oxford units dissolved in 750 c.c. of sterile isotonic saline solution. At the end of each day, blood was drawn for determination of the serum penicillin content.

TABLE II. PENICILLIN SERUM LEVELS FOLLOWING CONTINUOUS INTRAMUSCULAR ADMINISTRATION

DAILY PENICILLIN IN OXFORD UNITS (x 1,000)	AVERAGE SERUM LEVEL UNITS PER C.C.
500	0.35
1,000	0.6
2,000	1.5
3,000	2.0
5,000	5.0

*Results:* The average serum penicillin levels are given in Table II. Individual determinations varied as much as 100 per cent from the norm. In the same patient, under identical experimental conditions, serum penicillin concentrations, following the continuous intramuscular drip, tended to be consistently lower than those obtained by the continuous intravenous drip (Fig. 1).

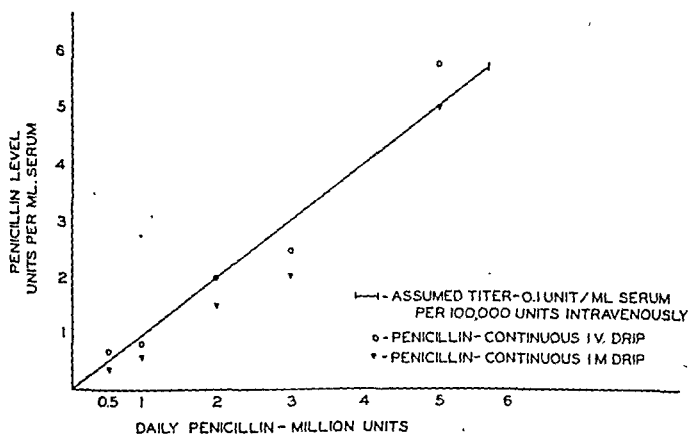


Fig. 1.—Comparison of serum penicillin levels following continuous intramuscular and continuous intravenous infusions.

*Comment:* The method of continuous intramuscular infusion is technically simple in so far as its administration is concerned. However, its maintenance, without serious complaint from the patient, is another matter. In our experience, the thigh muscles of the average patient tolerated only 750 c.c. of solution

per day. More than this amount almost invariably resulted in acute muscular soreness, with or without accompanying fever, and necessitated discontinuance of the infusion despite the fact that the site of the drip was changed from one thigh to the other each day. It was almost impossible to regulate the flow of less than 750 c.c. daily in a satisfactory manner.

*Serum Penicillin Concentrations Following Continuous Intravenous Drip.*—Rantz and Kirby<sup>3</sup> have shown that when penicillin is administered, at a constant rate, by continuous intravenous drip and in dosages up to 20,000 units per hour (480,000 units per day), predictable plasma levels can be obtained approximating 0.1 unit per cubic centimeter of plasma per 100,000 units of penicillin per day. We have extended these studies to include penicillin serum concentrations following continuous intravenous infusion in dosage up to 10,000,000 units per day.

*Results:* The results of serum penicillin assays are noted in Table III. In general, the values obtained are consistent with those anticipated, that is, 0.1 unit per cubic centimeter serum for each 100,000 units per day. It should be noted, however, that there is considerable variation in individual levels on different patients and even in the same patient at different times. These variations are due to fluctuations in the rate of flow of the intravenous solution and to mutations in the physiologic activity of the patients from time to time.

TABLE III. PENICILLIN SERUM LEVELS FOLLOWING CONTINUOUS INTRAVENOUS INFUSION

DAILY PENICILLIN IN OXFORD UNITS (x 1,000)	AVERAGE SERUM LEVEL UNITS PER C.C.	EXPECTED LEVEL UNITS PER C.C.	PER CENT DEVIATION FROM EXPECTED LEVEL
200	0.20	0.20	0.0
250	0.27	0.25	+ 8.0
300	0.34	0.30	+13.3
500	0.57	0.50	+14.0
900	0.90	0.90	0.0
1,000	0.91	1.0	- 9.0
2,000	2.0	2.0	0.0
3,000	2.6	3.0	-13.3
5,000	5.8	5.0	+16.0
10,000	9.2	10.0	- 8.0

*Comment:* The method of continuous intravenous drip is technically more difficult than fractional or continuous intramuscular dosage. However, the use of 23-gauge needles and the utilization of veins about the wrist, forearm, and lower extremities circumvent many inherent disadvantages. The method is attended with minimum discomfort to the patient and is extremely well tolerated. The incorporation of heparin in the venoclysis or its subcutaneous<sup>15</sup> deposition are valuable adjuncts for continuous intravenous therapy.

With meticulous attention to detail and the conjoint use of heparin, it is possible to keep the drip at a single site for as long as fourteen days. It is preferable, however, to change the site every three or four days to prevent undesirable local irritation, endophlebitis, or an occasional pyrogenic reaction.

The high serum levels make it possible to attack infections heretofore considered inaccessible to penicillin therapy. The combination of massive dosage schedules with such potentiating substances as para-aminohippuric acid<sup>16</sup> may yield even higher serum levels. Extreme resistance of an infecting agent should no longer be a deterrent to therapy; rather it should be a stimulus to vigorous, intensive attack.

It is significant to note that massive dosage up to 10,000,000 units per day is not attended with any symptoms of toxicity, either immediate or delayed.

Patients have been observed for months following massive dosage without any evidence of tissue damage.

#### CONCLUSIONS

1. Comparative studies of fractional intramuscular, continuous intramuscular, and continuous intravenous administration of penicillin have been presented.
2. Fractional intramuscular therapy is recommended for the treatment of acute, short-lived infections. Dosages should be given every two hours.
3. The continuous intravenous drip of penicillin yields serum levels superior to those obtained following continuous intramuscular injection.
4. Predictable serum penicillin levels are obtained following continuous intravenous drip in daily dosage schedules up to 10,000,000 units.
5. Daily dosages of penicillin up to 10,000,000 units are nontoxic.
6. The relation of these observations to the treatment of infections is indicated.

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# THE ACTIVITY OF PENICILLIN AT TEMPERATURES ABOVE 37° C.

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IT IS well known that compounds which inhibit bacterial growth exhibit an increase in efficiency as the temperature of incubation is raised. Eagle and Musselman\* have shown that the spirocheticidal effect of penicillin is enhanced by increasing temperatures. That this relationship holds for the action of penicillin upon *Staphylococcus aureus* is shown by the following observations.

Varying amounts of calcium penicillin were distributed in matched colorimeter tubes, and sufficient broth was added to bring the volume up to 1 c.c. The inoculum consisted of 4 c.c. of dextrose broth seeded with an eighteen-hour culture of *Staph. aureus*. The tubes were read in a Klett-Summerson photometer before and after incubation, the difference between the two values being taken to represent the relative bacterial growths. The period of incubation necessary to produce a suitable turbidity was related to the size of the inoculum, 1 c.c. of culture added to 100 c.c. broth giving reliable readings in three hours. Use of a smaller inoculum extended the incubation period to five or six hours but did not influence the results of the test. The observations have been repeated six times. On each occasion duplicate titration curves were prepared for each of the temperatures tested. While the differences in activity were not always of the same magnitude, the order was always the same, the penicillin showing greater activity at the higher temperature. Typical data are recorded in Tables I and II. The results are shown graphically in Fig. 1.

TABLE I. 100 C.C. BROTH SEEDED WITH 1 C.C. EIGHTEEN-HOUR CULTURE OF STAPH. AUREUS; INCUBATION PERIOD, THREE HOURS

PENICILLIN		BROTH (C.C.)	INOCULUM (C.C.)	TURBIDITY	
O. U.	VOLUME (C.C.)			37° C.	41° C.
0	0	1	4	85	82
0.02	0.2	0.8	4	85	58
0.04	0.4	0.6	4	65	24
0.06	0.6	0.4	4	44	14
0.08	0.8	0.2	4	28	7
0.10	0.1	0	4	7	7

TABLE II. 100 C.C. BROTH SEEDED WITH 0.1 C.C. EIGHTEEN-HOUR CULTURE OF STAPHYLOCOCCUS AUREUS, INCUBATION PERIOD, SIX HOURS

PENICILLIN		BROTH (C.C.)	INOCULUM (C.C.)	TURBIDITY		
O. U.	VOLUME (C.C.)			37° C.	39° C.	41° C.
0	0	1	4	100	93	44
0.005	0.25	0.75	4	96	86	39
0.01	0.5	0.5	4	98	79	28
0.02	1.0	0	4	83	58	18

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\*Eagle, Harry, and Musselman, A. D.: The Spirocheticidal Action of Penicillin in Vitro and Its Temperature Coefficient, J. Exper. Med. 80: 493-505, 1944.

It is evident that incubation at 39 and 41° C. increases the activity of the penicillin as detected by turbidity measurement. In the series which received the smaller inoculum the growth at 41° C. was less than that obtained at lower temperatures, but when the relative inhibition is calculated, using the control tube as 100 per cent, it is seen that the order compares well with the sets showing heavier growth.

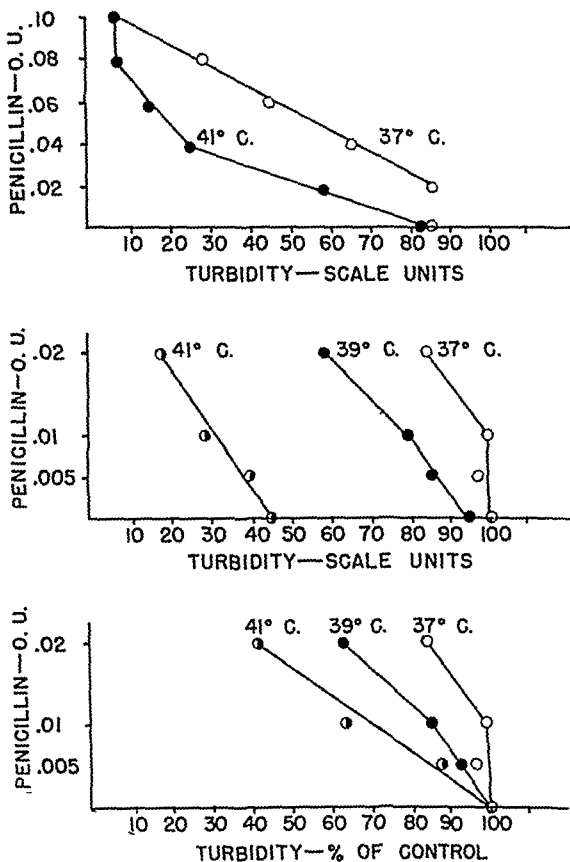


Fig. 1.

Two significant conclusions can be drawn from these facts. The combination of penicillin and fever therapy, suggested by Eagle and Musselman for the treatment of syphilis, may be expected to apply to other infections where penicillin alone is of value. A second application is in the estimation of penicillin levels using the measurement of turbidity as an index. By carrying out the incubation at 41° C., values of 0.005 Oxford unit can be detected with increased precision and accuracy, using *Staph. aureus* as the test organism.

## ORAL PENICILLIN IN THE TREATMENT OF GONORRHEA

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IT HAS recently been demonstrated by Free, Leonards, McCullagh, and Biro<sup>1</sup> that orally ingested penicillin is absorbed from the gastrointestinal tract in amounts that would be therapeutically effective. This finding has since been confirmed by several laboratories,<sup>2-7</sup> and obviously indicates the desirability of determining the effectiveness of orally ingested penicillin in the treatment of infections.

It has been shown that gonorrhea is particularly susceptible to treatment with penicillin,<sup>8</sup> a finding that has been confirmed by numerous clinical studies. Several recent reports have indicated that in uncomplicated gonorrhea better than 99 per cent of the cases responded favorably to treatment with penicillin.<sup>9-12</sup>

The present report describes studies carried out in a small series of patients with gonorrhea who were treated with orally administered penicillin.

### METHODS

All of the patients in the series were diagnosed as having gonorrhea by means of positive cultures and by clinical signs, symptoms, and history. The penicillin used was in the form of a dry powder contained in gelatin sleeve capsules. Each patient ingested a total of 1.6 million units of penicillin over a two-day period. The dosage schedule involved taking 100,000 units every two hours during the waking period. In all cases the patient was given the requisite amount of penicillin along with explicit instructions regarding the time and number of capsules to be ingested. The first eight patients indicated in Table I were not hospitalized, whereas Patients 9 through 14 were all treated during hospitalization. A complete forty-eight-hour urine sample was collected during the period of therapy, and the total penicillin excretion was determined by assaying the urine, using the cylinder plate method of Schmidt and Moyer.<sup>13</sup> Two urethral cultures in the male patients and two urethral and two cervical cultures in the female patients were obtained during the ten-day period following treatment, and in most of the patients a third culture was obtained at a somewhat later time.

### RESULTS

A total of fourteen patients with gonorrhea have been treated by the technique described. In all instances only negative cultures were obtained following treatment and all clinical signs and symptoms of gonorrhea disappeared. Table I shows the sex distribution of the patients and also presents the data for the urinary excretion of penicillin. The average excretion represents approximately 6 per cent of the ingested penicillin.

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No toxic effects were observed with this quantity of orally ingested penicillin, although one of the patients did complain of a certain amount of gastric distress and diarrhea. This effect could readily be due to the state of purity of the penicillin used in these studies since it was of a somewhat lower potency than most of the penicillin that is used for parenteral therapy. In all of the patients there was rapid improvement soon after therapy was begun and cultures taken three months after treatment in the first eight patients were all negative. In none of these patients has there been any recurrence of clinical signs or symptoms over a six-month period.

TABLE I

URINARY EXCRETION OF PENICILLIN (TOTAL OXFORD UNITS EXCRETED)				
PATIENT	SEX		CLINICAL RESULT	
1	M	92,000	Cure; 3	negative cultures after treatment
2	F	87,000	Cure; 3	negative cultures after treatment
3	M	110,000	Cure; 3	negative cultures after treatment
4	M	111,000	Cure; 3	negative cultures after treatment
5	M	166,000	Cure; 3	negative cultures after treatment
6	M	35,200	Cure; 3	negative cultures after treatment
7	M	63,000	Cure; 3	negative cultures after treatment
8	M	31,500	Cure; 3	negative cultures after treatment
9	F	112,500	Cure; 1	negative culture after treatment
10	F	74,000	Cure; 3	negative cultures after treatment
11	F	68,000	Cure; 2	negative cultures after treatment
12	F	70,000	Cure; 2	negative cultures after treatment
13	F	60,000	Cure; 2	negative cultures after treatment
14	F	59,000	Cure; 2	negative cultures after treatment

## DISCUSSION

In the present study the amount of penicillin employed was quite large but the results were uniformly successful. There is no reason to believe that this amount is necessarily required for successful treatment of gonorrhea, and there are considerations that indicate that a considerably smaller quantity would be effective. For instance, it has been found that with parenteral therapy 100 per cent successful results were obtained in 865 patients with gonococcal infection of the urethra who were treated with a total of 75,000 units given over a one-day period.<sup>12</sup> In half of our patients the amount of penicillin excreted in the urine was in excess of this amount. Certainly all of the penicillin that can be observed in the urine has been absorbed from the gastrointestinal tract. At the present time there is some indication that the amount of intestinal absorption can be increased by various means.

Since the completion of this work, a report has appeared by György and associates,<sup>14</sup> which describes the successful treatment of gonorrhea with oral penicillin. The findings of the present study offer confirmation of this observation.

Even though the quantity of penicillin required to treat gonorrhea and presumably other infections may be greater if the penicillin is given orally than if it is given parenterally, the convenience to the patient and the physician is such that with ample supplies of the drug available this appears to outweigh the added cost of the drug itself. This is especially true since for most parenteral therapy with penicillin it has been almost essential that the patient be hospitalized during the course of treatment. The use of various techniques to decrease the absorption of penicillin from the site of intramuscular injection

or to decrease the rate of urinary excretion of penicillin may obviate the need of hospitalization. However, the advantages of orally administered penicillin over these various other forms are quite apparent. The conditions of treatment employed with eight patients in the present study did not involve hospitalization and in these cases the patient took the medication merely following the instructions of the physician.

#### SUMMARY

Fourteen patients with gonorrhea, both men and women, have been successfully treated with penicillin administered by mouth. In no instance was the method of treatment unsuccessful. The advantages of orally administered penicillin in therapy are discussed.

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## POTENTIATION OF GERMICIDES WITH AEROSOL OT

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THE action of surface tension depressants in increasing (potentiating or synergizing) the activity of germicides and fungicides has received considerable attention in recent years.<sup>8-10, 12, 13, 15</sup> We have previously briefly noted the action of Aerosol OT (di-2-ethylhexyl sodium sulfosuccinate) in this respect.<sup>14</sup> The present paper gives quantitative data and deals with some of the factors involved in this action.

### METHODS AND MATERIALS

The method of the Food and Drug Administration known as "the F. D. A. method (special), *Staphylococcus aureus*, 37° C.,"<sup>11, 2</sup> was closely followed except for the use of sterile 0.2 c.c. Kahn pipettes to take 0.02 c.c. of medication mixture for inoculations. The reason for the use of the pipettes is that surface tension depressants such as Aerosol OT reduce the size of the drop (inoculum) taken on the standard loop from 0.02 c.c. to a much smaller volume, giving irregular results and making the potentiation appear to be greater than is actually the case. Details are given in our previous note.<sup>14</sup>

The culture of *Staph. aureus* used was No. 26 of our collection, which is No. 209 of the F. D. A. collection. Aerosol OT, when used, was always present in a concentration of 0.1 per cent in the medication tubes, a 1.0 per cent solution in distilled water being used in making up the dilutions. Concentrations are expressed in terms of grams of substance per 100 c.c. of solution. If allowed to stand for a day or so before use, the 1.0 per cent working solutions of Aerosol OT gave no bacterial growth when inoculated into the so-called "Reddish broth" used in cultivating the test organism in the F. D. A. method. Accordingly, no effort was made to sterilize the Aerosol OT working solution. This self-sterilizing action of 1 per cent Aerosol OT has been noted previously.<sup>9</sup>

The various dilutions of phenol were made from a solution of the pure compound standardized at 5.00 per cent by the method of the U. S. XI.<sup>3, p. 285</sup>

The cresol was Merck's U. S. P. grade. According to Merck's Index,<sup>4</sup> this is a mixture of ortho-, meta-, and para-cresols (in proportions not stated) and usually contains a few per cent of phenol. Although it is not a chemical individual but a mixture of compounds, it is nevertheless one of the purer forms of cresol commonly used in medicine and pharmacy. Since its density is almost exactly the same as that of water, the 1.00 per cent solution used in making the dilutions was prepared by pipetting exactly 2.00 c.c. into a 200 c.c. volumetric flask and making to volume with distilled water. The brown drops of cresol dissolved rather slowly to a clear solution. Although cresol is said to be soluble in about 50 parts of water,<sup>4</sup> attempts to prepare 2 per cent solutions were not successful, since a portion of the cresol persistently refused to dissolve.

The iodine was used in the form of tincture of iodine (strong), prepared in our laboratory according to the specifications of U. S. P. XI, containing 70

Gm. of iodine, 50 Gm. of KI, 50 c.c. of water, and made to 1 liter with 95 per cent ethyl alcohol. In making the dilutions in the medication tubes, a 1 per cent by volume aqueous solution of the tincture of iodine was used. In reporting the final results in terms of phenol coefficient, the limiting dilutions were recalculated into terms of concentrations *by weight* of tincture of iodine. Since the tincture had a specific gravity of exactly 0.900, the recomputation was easily done, although it would have been preferable to have made up the 1 per cent solution by weight rather than by volume.

The phenylmercuric nitrate was from Eastman Kodak Co., No. 3240. Exactly 1.000 Gm. was placed in a 2 liter volumetric flask and made nearly to volume. On heating and agitation in a water bath, the compound dissolved slowly. The solution was cooled to room temperature, then made to volume, giving a  $\frac{1}{2000}$  (0.05 per cent) working solution.

### RESULTS

Each phenol coefficient was run at least four times, both with and without Aerosol OT. It will be seen from Table I that 0.1 per cent Aerosol OT increases the germicidal action of phenol and cresol about 1.8 times (80 per cent increase). This increase was fairly uniform in different runs, which checked rather well with each other.

TABLE I. INCREASE IN PHENOL COEFFICIENTS OF GERMICIDES AGAINST STAPH. AUREUS AT 37° C. IN PRESENCE OF 0.1 PER CENT AEROSOL OT

GERMICIDE	PHENOL COEFFICIENTS		INCREASE IN PHENOL COEFFICIENT (OR BACTERIOSTATIC POWER)	
	GERMICIDE ALONE	GERMICIDE IN PRESENCE OF 0.1% AEROSOL OT	RATIO	PER CENT INCREASE
Phenol	1.0	1.8	1.80	80
Cresol, U. S. P.	2.4	4.4	1.83	83
Tincture of iodine (strong)	3.7-4.3	4.3-5.5	1.0-1.5	0-50
Phenylmercuric nitrate	166*	1300*	8.0	685

\*Bacteriostatic power only. True bactericidal power not determined.

With tincture of iodine, the results of different runs were very variable, whether Aerosol OT was used or not. It potentiated the germicidal action only slightly and irregularly, from no action at all to a 50 per cent increase when the lowest values for iodine are compared with the highest results for iodine plus Aerosol OT. When the iodine was added to the medication tubes, its brown color was partly discharged, undoubtedly because of chemical reaction with the constituents of the broth. This is in line with the well-known fact<sup>1</sup> that the phenol coefficient method is not particularly satisfactory when applied to preparations containing active halogen. It is also analogous to the findings of Gershenfeld and co-workers,<sup>9</sup> that Aerosol OT has little or no action in potentiating the germicidal action of hypochlorite (Zonite).

The most striking results were obtained with phenylmercuric nitrate. Although the results in different runs varied somewhat, it was nevertheless possible to determine fair average values, in which it was found that Aerosol OT increased the bacteriostatic power eightfold. It was not determined whether this represented an increase in true killing power or in bacteriostatic action only. It is possible that this great increase is due to the formation of a new compound in the solution, namely phenylmercuric di-2-ethylhexyl sulfosuccinate, since when 1 per cent solutions of Aerosol OT are mixed with 0.05 per cent solu-

tions of phenylmercuric nitrate in equimolecular amounts, a strong haze appears. Attempts to prepare such a compound in the dry state led to disappointing results, since the compound (if formed) appeared to decompose on drying. No further work is planned, but it seems probable that compounds of the phenylmercuric radical with the negative radical of appropriate surface active compounds might give germicidal or bacteriostatic compounds of great potency.

#### DIFFICULTIES IN THE F. D. A. PHENOL COEFFICIENT DETERMINATION

The usual difficulties connected with the current method of phenol coefficient determination were encountered. One of these was a reduced resistance of *S. aureus* to phenol. The limiting dilution (which would kill in ten minutes but not in five minutes at 37° C.) was frequently not 1-80, but 1-85 or 1-90. When this occurred, it was assumed that the resistance to other germicides diminished in essentially the same ratio, and the phenol coefficient was calculated under the conditions prevailing. Another difficulty was the familiar one of "skips," a higher dilution of germicide killing the test organism, while the next lower dilution failed to do so. This trouble was more common with iodine and phenylmercuric nitrate than with phenol and cresol. A discussion of the causes of these difficulties and irregularities must be left for a future paper.

#### CHARACTERISTICS OF AEROSOL OT

*Reduction of Surface Tension.*—To determine the reduction of surface tension by Aerosol OT, readings were taken with the DuNouy tensiometer. The following data represent the average of four determinations in each case, in terms of dynes: distilled water, 70.7; medication tube mixture, 1-90 phenol, 51.1; medication tube mixture, 1-100 phenol, 53.3, which diminished to 29.8 with 0.1 per cent Aerosol OT present. The 1.0 per cent Aerosol OT working solution had a value of 28.9 dynes. The potentiating action of Aerosol OT is presumably due, at least in part, to a reduction of the interfacial tension between the bacterial cells and the surrounding liquid, so that the germicide reaches or penetrates the cells more readily.

*Acidity of Old Aerosol OT Solutions.*—Solutions of Aerosol OT gradually become more acid with age. When freshly prepared, such solutions have a pH of from 6.3 to 6.9, closely approaching that of the distilled water used, since Aerosol OT has little or no buffering power. However, the solutions showed very little decrease in pH at first. One 5 per cent solution stored in the refrigerator for twenty-two days showed a pH of 6.90 by the glass electrode. Older solutions showed lower pH values. Of those held at room temperature, one at 4.47 per cent had a pH of 2.90 after sixteen months, while a 0.04 per cent solution had a pH of 5.79 after fifteen months. The acidity of these old solutions is undoubtedly due to partial hydrolysis of the Aerosol OT with liberation of acid. Aerosol OT solutions are unstable in the presence of any great amount of electrolyte, the Aerosol OT settling out in the form of droplets. Accordingly, efforts to buffer solutions of Aerosol OT at pH 7 were unsuccessful. The procedure of using reasonably fresh 1.0 per cent solutions of Aerosol OT, with a pH of not less than 6.30, was adopted. Due to the poor buffering power of Aerosol OT, such solutions gave no appreciable shift in pH when added to the liquid in the medication tubes, so that the potentiating action was not due to any diminution in pH but only to the reduction of interfacial tension between the liquid and the cells or to other factors.

*Increased Potentiation by Old (Acidic) Aerosol OT Solutions.*—In our earlier work, before the pH of the Aerosol OT solutions was properly controlled,



a somewhat greater potentiating action was obtained than with the nearly neutral solutions used later. A 1.0 per cent solution (prepared from the sixteen-month-old 4.47 per cent solution at pH 2.90) had a pH of 2.88, depressing the pH of medication tube mixtures containing phenol from 6.8 to about 5.2 to 5.4, while increasing the apparent phenol coefficient of the phenol by 100 per cent instead of 80 per cent. This increase in the activity of germicides with diminished pH is well known<sup>9, 11, 12</sup> and must always be taken into account in potentiation experiments. In certain applications it would be preferable to use potentiated germicide solutions at the lowest possible pH.

#### SUMMARY

Under conditions of nearly constant pH, 0.1 per cent Aerosol OT increases the phenol coefficient of germicides in the F. D. A. method (special), *Staph. aureus*, 37° C., as follows: phenol, from 1.0 to 1.8; U. S. P. cresol, from 2.4 to 4.4; U. S. P. tincture of iodine (strong), doubtfully and irregularly from 3.7-4.3 to 4.3-5.5. The apparent phenol coefficient of phenylmercuric nitrate (not corrected for bacteriostasis) is increased from 166 to 1,300. The characteristics of Aerosol OT as a potentiating agent for germicides are described.

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## MENINGOCOCCUS GROUPING

### NOTE ON EXPERIENCE WITH THE CAPSULAR SWELLING TEST

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FOR approximately forty years attempts have been made to differentiate strains of meningococci by means of serologic reactions. The early work of Kutscher (1906),<sup>1</sup> Dopter (1909),<sup>2</sup> Elser and Huntoon (1909),<sup>3</sup> and Arkwright (1909),<sup>4</sup> made use of agglutination and absorption tests. Similar methods used by later workers, Gordon and Murray (1915)<sup>5</sup> and Nicolle, Debains, and Jouan (1918)<sup>6</sup> enabled the investigators to divide the meningococci from epidemic and sporadic cases into four groups. Griffith (1917)<sup>7</sup> and Scott (1918)<sup>8</sup> divided them by agglutination into two main groups. Branham, Taft, and Carlin (1931)<sup>9</sup> made detailed studies of meningococci isolated in the United States and indicated the difficulties in attempting a serologic classification when separation into four types by agglutinin absorption was done.

A method of differentiation described by Clapp, Phillips, and Stahl (1935),<sup>10</sup> based on the capsular swelling exhibited by certain strains of meningococci with homologous serum (rabbit), has been strangely disregarded by laboratory workers, although Cooper and Walter (1939),<sup>11</sup> of the New York City Health Department, apparently had some success with it. Einhorn (1937)<sup>12</sup> described the application of the Neufeld reaction to the identification of types of meningococci. Branham called attention to it in *Diagnostic Procedures and Reagents* (1941) and more recently (1945)<sup>13</sup> advised its use.

Because of the increase in the number of cases of meningococcus meningitis in Massachusetts during the past two years, it was possible for the Bacteriological Laboratory of the Massachusetts Department of Public Health to evaluate this method. From Jan. 1, 1943, to April 30, 1945, a total of 195 specimens of spinal fluid was examined for meningococci. They were found in seventy-three specimens, of which sixty-four were spinal fluid and nine were cultures on blood agar plates or some medium on which the sender had made cultures of the spinal fluid. The majority of these specimens were received by mail and were several hours old when delivered at the laboratory. Autolysis of the meningococci did not appear so extensive as to prevent the cultivation of the organism in most instances where it was believed to have been present.

The laboratory procedure was as follows: Upon the receipt of the specimen, a Gram stain was made of the fluid, for orientation. If any gram-negative diplococci were seen on examination with the oil immersion objective at a magnification of 900 to 1000, a capsular swelling test was set up at once with the spinal fluid. If no organisms resembling meningococci were found, the spinal fluid was centrifuged for an hour and then the sediment examined by Gram stain. Quite often other bacteria such as *Hemophilus influenzae*, pneumococci, or streptococci were found and appropriate tests were made for their identifica-

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tion. Even if no bacteria were seen in the sediment, a capsular swelling test for meningococci was set up, as a diplococcus overlooked in the stained preparation might thereby become apparent.

The capsular swelling test was made in a manner similar to that used for the Neufeld reaction in pneumococcus typing. A loopful of spinal fluid or sediment was placed on a plane slide and to it were added one loopful of the meningococcus Group 1 monovalent rabbit serum and a loopful of Loeffler's methylene blue. The mixture was stirred and a cover glass ( $\frac{7}{8}$  inch square, No. 1) was placed over the wet preparation. Another mixture was made using Group 2A serum. The meningococcus sera used were obtained from the Lederle Laboratories. Cultures received on solid media were rubbed up in a drop of normal saline solution when the test was made. These preparations were examined with the oil immersion objective and the light partially dimmed. They were thoroughly examined when first made and if no capsular swelling was observed, they were re-examined after an hour. In a positive reaction the capsule around the blue-stained diplococcus was enlarged, was of a greenish-gray color or ground-glass appearance, with a thin dark line at the periphery. This reaction was seen with meningococci of Group 1 (types 1 and 3) and of Group 2A; up to the present time, no reaction has been observed with the Group 2 or Group 4 strains. If a reaction was seen with the direct preparations of the fluid or sediment, a report of Group 1 or Group 2A meningococcus was telephoned at once to the sender. The capsular swelling with the homologous antiserum (rabbit) seemed to be a specific reaction, for in every instance the cultural, agglutination, and repeated quelling tests confirmed the original diagnosis. If no reaction was seen in the direct preparations of the fluid or sediment, cultures were made from the sediment on chocolate agar, starch agar, Loeffler's blood serum, Avery broth, etc. in duplicate and incubated at 34° C. One set was incubated in the air and the other in an atmosphere containing about 10 per cent carbon dioxide, satisfactorily obtained in a "candle jar."

Occasionally mouse inoculation was used, especially when no organisms were found on microscopic examination. The technique employed was Miller's mucin-mouse method as described by Sulkin (1939).<sup>14</sup> This method may enable one to demonstrate capsular swelling within a few hours after the intraperitoneal inoculation of the mouse.

The cultures were examined for bacteria after from eighteen to twenty-four hours' incubation and thereafter every day for several days. Whenever any gram-negative diplococci resembling the meningococcus were seen in any culture or mouse peritoneal exudate, capsular swelling tests were made.

In this study, of the seventy-three specimens in which meningococci were found, fifty-four (74 per cent) were grouped directly without waiting for cultural growth; the remainder (26 per cent) grouped after incubation of the cultures, usually on the following day. There were sixty-five strains of meningococci belonging to Group 1 and five to Group 2A. Three specimens contained Group 2 meningococci which could be grouped only by agglutination tests.

Results which were doubtful were obtained (1) in the examination of specimens which were obviously contaminated when received, usually in non-sterile containers (because of overgrowth of spore-bearing bacilli or staphylococci, a few meningococci might have been obscured); (2) in the examination of specimens in which an occasional gram-negative diplococcus was seen in the

spinal fluid which could not be cultivated and showed no capsular swelling. However, if an occasional meningococcus is seen with a swollen capsule, that would appear to establish the identification without subsequent cultivation.

The simplicity of the capsular swelling test should make the grouping of meningococci, to which it is adaptable, a common practice in laboratories, so that the occasional need of the auxiliary use of homologous monovalent anti-meningococcal serum can be met with as little delay as possible.

These examinations were made by the bacteriologists of the Bacteriological Laboratory of the Massachusetts Department of Public Health, to whom the writer is greatly indebted.

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## THE DIAGNOSTIC SIGNIFICANCE OF INCLUSION BODIES IN RABIES AND CANINE DISTEMPER

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IN A LARGE island base in the South Pacific Area, rabies has never been known to occur. An analysis of communicable diseases existing on this island by the local health authorities reveals a complete absence of rabies. Due to the increased war shipping and the opening of this area as an important harbor, together with the introduction of dogs from the continental United States, the problem of rabies loomed on the epidemiologic horizon.

The incidence of canine distemper in this island base is extremely high. Native laboratory workers have informed us that the incidence is as high as 60 per cent among the native dogs. These dogs present the respiratory, gastrointestinal, and urinary findings so characteristic of the disease. We have, during the course of an investigation for the presence of rabies, found an encephalitis complicating canine distemper. This has produced nervous symptoms and signs almost identical with the clinical picture seen in rabies. Inclusion bodies, similar but not characteristic of the Negri bodies found in rabies, have been found in various portions of the brain. We present this paper to indicate the simulation of canine distemper to rabies and its importance in differential diagnosis.

Two dogs, who had been pets of a marine group situated on this island base, suddenly became restless, excitable, and vicious. They presented focal epileptic convulsions and had bitten six marine soldiers. No history could be elicited regarding respiratory and gastrointestinal findings. The marines bitten were given the Pasteur treatment. One dog was immediately shot by the Marine personnel and sent to a neighboring Naval hospital for autopsy examination. They amputated and buried the head of the second dog.

*Protocol FH-A1.*—At autopsy, the body externally revealed no diagnostic findings. The heart revealed slight congestion. No valvular lesions were visible. The lungs revealed a hemorrhagic consolidation, focal in distribution, throughout the left lower lobe. Raised, thickened gray-white nodules which, on close examination, consisted of thickened bronchiolar walls were present. A thick mucoid exudate could be expressed from the cut surfaces. The surrounding lung tissue was edematous, boggy, and congested. The remaining viscera were intact. The brain was not weighed. The meninges and the brain substance revealed congestion and moderate edema. Coronal sections throughout the brain revealed congestion of the small vessels, with petechial hemorrhages throughout. Tiny petechial hemorrhages, measuring 2 to 3 mm. in diameter, were distributed through the motor cortex, the mid-brain, pons, medulla, hippocampus, and cerebellum. Touch preparations from these areas were made.

They were stained by Mann's and Seller's methods. Smears from the motor cortex, medulla, and cornu ammonis revealed oval bodies, measuring 2 to 10  $\mu$  in diameter, occurring singly or multiple (three to five) in the cytoplasm of the ganglion cells. The cytoplasm stained a pink-red with Mann's stain and revealed small clear vacuolated-like bodies in its interior. No characteristic central basophilic chromatin granulations were visible however. At this time, these were interpreted as Negri bodies, and a diagnosis of rabies was made. Hematoxylin-eosin stained sections from various portions of the brain revealed a moderate edema, with moderate congestion and hemorrhage. Slight perivascular mantling with small lymphocytes and polymorphonuclear leucocytes was found most prominent in the mid-brain. Tiny focal areas of nerve cell degeneration, characterized by swelling of cytoplasm and nucleus, in turn, surrounded by an early glial proliferation were present. Sections stained by Mann's stain revealed identical inclusion bodies. The picture did not fit the morphologic interpretation of rabies, but the inclusion bodies appeared strongly indicative of such a diagnosis. Animal inoculation was not performed at this time.

As a result of this preliminary investigation, the head of the second dog was disinterred and turned over to an Army general hospital laboratory for verification of diagnosis.

*Protocol GII-A1.*—Examination of the brain revealed moderate congestion and edema of the brain substance and overlying meninges. There was moderate autolysis throughout. Coronal sections throughout the brain revealed vascular congestion with petechial hemorrhages. Touch preparations, utilizing the method described, revealed similar inclusion bodies in the ganglion cells of the motor cortex, the temporal lobe, the hippocampus, pons, and mid-brain. They were also found in the Purkinje cells of the cerebellum. Histologic examination revealed an intense congestion with perivascular hemorrhages, in turn surrounded by focal areas of degeneration, characterized by nuclear pyknosis and swelling of the cell cytoplasm. In some areas, there were perivascular collars of lymphocytes and neutrophilic leucocytes. These were most prominent in the region of the mid-brain and medulla. Intracerebral inoculations of a saline suspension of brain tissue, including motor cortex, cornu ammonis, and cerebellum, were performed, utilizing a guinea pig, mouse, and rabbit, respectively. Leach's methods with slight modifications were employed. The animals were watched for a period of sixty days. They at no time presented clinical findings suggestive of rabies, and at the end of this period were alive and healthy.

The possibility that we were not dealing with true rabies but with an encephalitis, probably viral in origin and associated as part of the clinical syndrome of canine distemper, was now entertained. Fortunately, a member of the Veterinary Corps had brought to the laboratory four dogs which were clinically diagnosed as having distemper but which presented, in addition to the respiratory and gastrointestinal findings, symptoms suggestive of brain involvement. These dogs were watched. They died within a period of seventy-two hours. The autopsy findings in each of the cases were almost identical. We present one such representative case.

*Protocol GII-A3.*—The dog presented a mucopurulent discharge in both nostrils. The right and left pupil each measured 4 mm. in diameter. No other noteworthy findings were noted on external examination. The heart presented no remarkable features. The lungs were congested and edematous. There were irregular focal areas of deep blue-red consolidation measuring 3 to 4 cm. in

diameter and raised above the general surface for a varying distance. These were surrounded by blue-gray depressed areas of atelectasis. These were most prominent in the right and left lower lobes. The bronchiolar walls were thickened, and a thin mucopurulent exudate exuded from the cut surfaces. The larynx and trachea revealed an edematous mucous membrane. Scattered throughout were numerous irregularly distributed petechial hemorrhages. There was a thin mucopurulent exudate in the lumen. The bronchi and bronchioles were congested and edematous. The walls were thickened and indurated and contained a semiviscid gray-white exudate. The bladder presented numerous petechial hemorrhages throughout the mucosa. A number of these measured up to 1 cm. in diameter. There was no evidence of trabeculation or diverticula formation. The remaining viscera presented no prominent findings. There was congestion of the cerebral meninges. The brain substance itself was edematous and congested. Coronal sections throughout revealed a petechial hemorrhage, most prominent in the mid-brain and medulla, associated with focal areas of gray-yellow degeneration. Touch smears from various sections of the brain revealed identical inclusion bodies to those found in the previous cases. Touch smears from the trachea, bronchi, and mucosal wall of the bladder revealed similar inclusion bodies. Microscopic examination revealed findings in the brain similar to those described in the previous two protocols: namely, a moderate congestion, with perivascular collaring of lymphocytes and neutrophilic leucocytes, focally distributed petechial hemorrhages, and areas of nuclear pyknosis and cytoplasmic swelling surrounded by a zone of neuronophagia. Bacterial stains with the Brown-Brenn<sup>1</sup> method revealed no organisms. Microscopic examination of sections from the right and left lower lobes revealed extensive bronchiolar dilatation with desquamation and ulceration of the mucous membrane. The lumen was filled with a combined mononuclear and polynuclear cellular exudate. The bronchiolar and alveolar walls revealed a round cell infiltration. Small numbers of alveoli contained scattered monocytes and lymphocytes. Alveolar hyaline membranes were not visible. The alveolar septa were thickened and infiltrated with round cells. There was congestion of all the blood vessels, including the alveolar capillary bed. An occasional inclusion body was seen in the remnant epithelial lining of the small bronchioles.

Intracerebral inoculation of a guinea pig, rabbit, and white mouse, respectively, with sterile saline emulsions of involved brain tissue, produced no effect after sixty days.

The remaining three cases gave identical findings on macroscopic and microscopic examination and with animal inoculation.

#### COMMENT

The cases presented are of diagnostic and epidemiologic importance. It is apparent that we were not dealing with rabies but with canine distemper or a variant of this disease complicated by an encephalitis. Nervous symptoms due to encephalitis are apparently not new findings in canine distemper. Zinsser and Bayne-Jones<sup>2</sup> record this in their discussion of distemper of dogs. The finding of inclusion bodies in the ganglion cells of the brain in meningo-encephalitis, secondary to canine distemper, has been recorded by Kelser.<sup>3</sup> It appears therefore that any inclusion bodies found in the brain are not directly diagnostic of rabies. Negri bodies have a distinct specific structure with the central basophilic staining chromatin granulation, a prominent finding. It is not meant to infer that prophylactic and therapeutic measures

should not be undertaken but rather that careful watching of the dog be performed; that search for the specificity of the inclusion bodies be made; that animal inoculation be performed in all instances for confirmation; and should the animals be brought to autopsy, scrapings of the trachea, bronchi, and urinary bladder be made in order to rule out canine distemper with secondary encephalitis.

#### SUMMARY AND CONCLUSIONS

1. Six dogs are described with nervous symptoms suggestive of rabies but with morphologic evidence of an encephalitis.
2. In all cases, inclusion bodies, similar but not characteristic of Negri bodies, were found in the ganglion cells of the cerebrum, mid-brain, pons, medulla, and in the Purkinje cells of the cerebellum.
3. In five dogs, there was evidence of a "virus" pneumonia. Similar inclusion bodies were found in tracheal, bronchial, and vesical smears in four of these cases.
4. Intracerebral inoculation of guinea pigs, mice, and rabbits, utilizing a sterile suspension of involved brain tissue from five of these cases, did not confirm a diagnosis of rabies.
5. Canine distemper with complicating encephalitis may simulate rabies clinically and morphologically.

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#### OCULAR DISTURBANCES IN RIBOFLAVIN DEFICIENCY

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EARLY Italian writers on pellagra, notably Soler<sup>1</sup> in 1791, included, among other symptoms of the disease, inflammation of the cornea and corneal opacities. In the light of present knowledge, these lesions might be interpreted as manifestations of riboflavin deficiency.

That certain ocular conditions might be due to a deficiency of one of the B complex vitamins ( $B_2$  or G) was suggested by observations on rats maintained on a diet low in this vitamin. Between 1928 and 1931 numerous investigators<sup>2-4</sup>

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referred to ophthalmia and conjunctivitis observed in rats deficient in vitamin B<sub>2</sub> or G. In 1931 Day, Langston, and O'Brien<sup>5</sup> reported alopecia of the eyelids, lacrimation, conjunctivitis, thick, swollen, inflamed lids, cataract, and anterior interstitial keratitis in rats on a vitamin G-deficient diet. These findings were confirmed by a number of investigators. In 1937 Day, Darby, and Langston<sup>6</sup> reported that pure riboflavin, natural or synthetic, would prevent the keratitis, cataract, and other ocular manifestations of vitamin G deficiency. The following year the same laboratory reported that the parenteral administration of pure riboflavin to rats with ocular manifestations of riboflavin deficiency resulted in the rapid disappearance of the keratitis and in many instances the arrest of the cataractous changes. Day and Darby<sup>7</sup> in 1938 reported that severe keratitis was seen in all animals receiving a limited amount (30 gamma) of pure riboflavin weekly but that cataract was less common than in animals receiving a diet from which riboflavin was rigidly excluded. Early in 1939 Bessey and Wolbach<sup>8</sup> described their important observations on the cornea of rats whose diets were deficient in riboflavin. In their summary of a very careful histologic study of the vascularization of the cornea of the rat with riboflavin deficiency, Bessey and Wolbach stated: "Vascularization of the cornea of the rat in the absence of antecedent pathology is probably a specific and the most reliable criterion of riboflavin deficiency." A little later Eckhardt and Johnson<sup>9</sup> reported ocular changes in rats on a riboflavin-deficient diet. They stated, "Keratitis and vascularization of the cornea were more consistent ocular changes than cataract and were improved by the addition of riboflavin to the diet."

The first definite statement in regard to certain eye symptoms in persons with riboflavin deficiency seems to be that of Spies, Bean, and Ashe in May, 1939.<sup>10</sup> They stated that patients with riboflavin deficiency complained of visual disturbances which disappeared within forty-eight hours following the administration of riboflavin and that these symptoms returned within from ten to twenty days after riboflavin was discontinued. This statement was amplified in a paper published by Spies, Vilter, and Ashe in September, 1939,<sup>11</sup> in which attention was called to heretofore undescribed ocular manifestations of riboflavin deficiency in human beings. These manifestations included bulbar conjunctivitis, dilatation of the conjunctival vessels, burning of the eyes, lacrimation, failing vision, and extreme photophobia. These symptoms, in many persons, disappeared following riboflavin therapy. Soon many articles appeared describing various ocular manifestations associated with riboflavin deficiency. In November, 1939, Sydenstriker, Geeslin, Templeton, and Weaver<sup>12</sup> reported that conjunctivitis and photophobia in a patient with riboflavin deficiency improved following riboflavin therapy. In January, 1940, Kruse, Sydenstriker, Sebrell, and Cleckley<sup>13</sup> reported ocular lesions in nine patients with other symptoms of riboflavin deficiency and stated that the principal manifestation was keratitis. Following the administration of riboflavin, the corneal lesions improved or disappeared and reappeared on cessation of riboflavin therapy. In May, 1940, Johnson and Eckhardt<sup>14</sup> reported that in a series of thirty-six patients with rosacea keratitis, only four failed to respond satisfactorily to oral riboflavin therapy. In June, 1940, Sydenstriker and his associates<sup>15</sup> again reported on the visual changes associated with riboflavin deficiency. They stated that photophobia, dimness of vision, and impairment of visual acuity were relieved promptly by riboflavin therapy, in some instances before visible changes occurred in the cornea. Using the slit lamp in examination of the cornea, they observed that proliferation and engorgement of the limbic plexus was the earliest

lesion. This progressed to superficial vascularization of the cornea and the production of interstitial keratitis. They found that congestion of the sclera, vascularization, opacities of the cornea, and abnormal pigmentation of the iris responded rapidly to the administration of riboflavin.

Numerous reports have continued to appear in the medical literature, some describing various ocular manifestations associated with riboflavin deficiency and others denying their existence. Today there is considerable controversy on the subject, and a wide divergence of opinion exists among investigators in the field. Some physicians doubt that riboflavin deficiency affects the eyes, whereas we, as well as many other investigators, believe that it gives rise to ocular lesions and that a great many persons are affected. It is easy to see the differences in the point of view between the two groups. It is most difficult to overcome them, but success in doing so is vital to proper medical care.

It is hoped that this paper will aid the physician in the recognition and treatment of the ophthalmic lesions of riboflavin deficiency. It is concerned only with the study of ocular lesions in patients whom we believe to have riboflavin deficiency. This study, which was begun in 1938 and extended through 1944, is based entirely on naturally occurring lesions which responded to riboflavin therapy.

In 1938 and 1939 we had conferences with Dr. M. A. Blankenhorn, Professor of Medicine, and Dr. Derriek Vail, Professor of Ophthalmology, University of Cincinnati, in regard to using the slit lamp in examining patients in the Nutrition Clinic of the Hillman Hospital in Birmingham, Ala. As a result of these conferences, Dr. William Ashe and Dr. Barnet Sakler came to Birmingham for a short period of study, Dr. Ashe to aid in general medical aspects of the problem and Dr. Sakler to examine the cornea. Dr. Sakler noted the same symptoms we had observed and thought that the cornea should be studied in a considerable number of subjects to determine the relationship of riboflavin to vascularization of the cornea. He believed the problem required extensive and intensive collaboration of both ophthalmologists and physicians interested in nutrition; accordingly, Dr. Vail sent Dr. K. W. Ascher, of the Department of Ophthalmology of the University of Cincinnati, to our Nutrition Clinic at the Hillman Hospital in Birmingham in 1940 and 1941. A report by Dr. Vail and Dr. Ascher was published in the *Transactions of the American Ophthalmological Society*, Seventy-Eighth Annual Meeting, 1942.

The collaboration with the Department of Ophthalmology can be summarized by stating that the ophthalmologists were, by training, skillful in the use of the slit lamp and in the interpretation of the ocular findings, whereas our general knowledge of experimental medicine and nutritional deficiency diseases enabled us to point out general features which otherwise might have been disregarded. Out of this collaboration has come a much more logical development of the study than could have been achieved without it.

#### METHOD OF STUDY

In the Nutrition Clinic we examine several thousand persons each year. Many of these persons have no clinical evidence of dietary deficiency disease. If, from their dietary and medical history, we can discover no reason for their having a nutritional deficiency and if the physical examination reveals no evidence of deficiency, they are not registered as patients in the Nutrition Clinic but are referred elsewhere for diagnosis and treatment. In some instances they return to their own physicians and in other instances to other departments of the Hillman Hospital. Those who have no diagnostic evidence of dietary

deficiency at the time but whose histories and symptoms suggest that they might be developing dietary deficiency disease are observed frequently. These persons and those with clinical dietary deficiency diseases are carefully studied to obtain all possible information on their past and present nutritional status and health by means of thorough medical and dietary histories, repeated physical examinations, and indicated laboratory determinations. A detailed description of the methods we have found useful in obtaining such information was published recently.<sup>10</sup> The dietary histories revealed that 90 per cent of the patients who came to the Nutrition Clinic had eaten diets of varying degrees of inadequacy. In analyzing the data obtained from the medical histories and physical examinations, we found that 70 per cent of the patients having clinical deficiency disease complained of visual disturbances.<sup>11</sup> From this group in 1938 we began selecting patients for study of their ocular symptoms.

We felt that more comparable cases could be selected if the group of physicians selecting them was not too large and if there were frequent consultations among the physicians concerned. Accordingly, all the cases were selected by Dr. Richard W. Vilter, by Dr. William B. Bean, and by us. The criteria for the initial selection of patients were:

1. Ingestion of a diet inadequate in riboflavin
2. No apparent means of changing the diet significantly for a number of years
3. History of one or more of the following symptoms: burning and itching of the eyes, failure of visual acuity, photophobia, lacrimation, conjunctivitis, night blindness
4. Ocular lesions characterized by abnormal dilatation of conjunctival and corneal blood vessels and, in many instances, by corneal ulceration

Since our interest was concerned primarily with endemic riboflavin deficiency, we excluded most of the patients with known organic disease. We did include ten persons with hypertensive heart disease and twelve who had trauma to some part of the body other than the eyes. All persons with traumatic disease of the eyes were excluded, as were all persons who had syphilis, tuberculosis, chronic alcoholic addiction, diabetes mellitus, and any known disease of the alimentary tract. Many persons with ocular lesions also had clinical evidence of advanced beriberi, pellagra, or scurvy which required immediate and intensive treatment. These persons were likewise excluded from this special study as we wished to eliminate the possibility that the treatment given for one of these coexisting deficiency diseases might affect the natural course of the ocular lesions and their response to riboflavin therapy. We have observed that this can occur.

We freely admit that we do not know what specific ocular symptoms and lesions are caused by riboflavin deficiency. Nevertheless, that many such manifestations do respond to riboflavin therapy must be granted. It is only by determining the effect of riboflavin therapy on these ocular manifestations that a diagnosis of riboflavin deficiency can be made. As can be seen from the criteria used for selecting the patients for this particular study, we made every attempt to exclude persons whose eye involvement might be due to other causes. That this method of diagnosis and treatment is unsatisfactory we know, but until more reliable methods are available, we can recommend no better way of relieving and often rehabilitating persons in whom ocular disturbances of this special type have developed.

After excluding the above groups, we arbitrarily selected patients until we had 500. Two hundred with moderate eye involvement were given riboflavin

by mouth only. Three hundred with more severe eye involvement were given riboflavin at least once intravenously. It is with this group of 300 patients that this report is concerned.

Some of these patients were admitted to the hospital, but the majority remained at home and came to the clinic for observation and treatment. They were observed before, during, and after therapy. In many cases, slit-lamp examinations were made. Bacteriologic studies were made in fifty patients. These included smears and cultures taken from the lesions and from the exudate from the eyes. The blood level of riboflavin was determined in fifty patients. Urine examinations were done on the first 150; then it was found that the urinary excretion of riboflavin was so low that it could not be detected, and it did not seem worth while to study it in additional patients. A few of the early determinations of riboflavin in the urine were made by Dr. William Ashe, using the Emmerie method.<sup>17</sup> Most of the urine examinations were made by Dr. S. S. Sanders, who also made all the blood determinations, using the microbiologic technique of Snell, Strong, and Peterson.<sup>18</sup>

The patients in this study included males and females, both white and Negro, ranging in age from 8 to 92 years. Throughout the course of the study, we obtained a dietary history at frequent intervals and made repeated physical and eye examinations.

#### OBSERVATIONS

The dietary histories obtained at frequent intervals revealed that, while a few patients had eaten good diets until only a year prior to the onset of illness, most of them had subsisted on inadequate diets for many years. In most instances the income was low, and in order to have enough food to satisfy hunger, they had purchased low cost foods, such as cornmeal, flour, dried beans, sugar, and fat pork, foods that are relatively inexpensive. Higher cost foods, such as milk, meat, eggs, green vegetables, and fruit, either were never included or were included at infrequent intervals. In a comparatively small number of cases, loss of appetite or food idiosyncrasies were responsible for the patients' having had inadequate diets. Routine nutrition histories, valuable as they are in suggesting whether or not the diet has been adequate, do not indicate the degree of the deficiency. Accordingly, in 100 of the 300 patients, we made repeated detailed studies of the diet. Observation of these patients over a long period of time convinced us that we could rely on the information obtained concerning their diets. Patients were instructed to keep a daily record of their food intake for a week at different seasons of the year. During these times, frequent visits were made to their homes to check their food supply and to determine how their food was prepared and served. From these data, the average daily intake of nutrients was calculated. The results of this study are shown in Fig. 1. Note that the diets of these patients were generally deficient and that they supplied only 36 per cent of the recommended allowance of riboflavin.

The most conspicuous and consistent early complaint elicited from analysis of the medical histories was that of weakness and fatigability. Frequently these patients were unable to participate in any activity and most of them had been unable to work regularly for years. A few of them had been able to work for a few hours and then were exhausted for a number of days. Very common complaints were nervousness, insomnia, headache, burning and aching of the legs and feet, burning and cramping of the stomach, constipation, burning of the skin over various parts of the body, itching and burning of the eyes, and failing

Recently she has had more bleeding from the hemorrhoids and she has been referred to the surgical clinic. Blood studies made by Mrs. Calvin Koch and Mrs. Hayes Caldwell show that within the last five months the patient's red blood cell count has changed from 4.1 to 3.6 million per cubic millimeter and the hemoglobin from 8.8 to 8.2 Gm. per 100 c.c.

This case has been included for several reasons. In the first place, it is the only one in which it was necessary to do a local operative procedure on the eye. In the second place, this woman is one of four patients who, as far as we know, had no organic disease at the time she was selected for study but who later developed one. In the third place, she has an anemia which must be treated with iron, and we must determine whether or not she has liver disease or just what condition is causing the bleeding hemorrhoids. This case is so much like some we have seen in the General Hospital in Cincinnati where organic disease plays a more prominent role than it does among patients in the Nutrition Clinic in Birmingham that it seemed wise to include it.

We regard the data presented merely as a progress report. Despite the fact that by giving riboflavin we have been able to stop the severe ulcerative process in the cornea and have been able to rehabilitate the patient to the place where she can work regularly as a cook in a restaurant, we will not consider her completely rehabilitated until the hemorrhoids and anemia are successfully treated and until she improves her diet so that it is no longer necessary to give her riboflavin to prevent frequent recurrences of her ocular disturbances.

CASE 3.—L. N., a 68-year-old Negro woman, was brought to the Nutrition Clinic in February, 1941, complaining of excruciating pain in her eyes. She was unable to see well enough to walk unaided. She was admitted to the Hillman Hospital for diagnosis and treatment.

Family history and past history were irrelevant.

Present illness: She was in good health until six years prior to coming to the clinic. At this time she began having dizziness and shortness of breath. She consulted a physician who advised her to stop working. Until this time she had always had a good appetite and her diet had been liberal and varied, although it was high in carbohydrate. After she gave up her work as a laundress her only income was from a daughter who sent her small sums of money from time to time. It never averaged more than eight or nine dollars a month. With the money she had left, after paying for fuel and rent, she could buy little but corn meal, flour, syrup, dried beans, sweet potatoes, and fat pork, so she ate large amounts of these foods. She never had lean meat or milk and seldom had eggs or green vegetables. For six years her diet had been inadequate in all nutrients except calories (Fig. 4). Two years prior to the time she came to the clinic her eyes began to burn and "water" and she noticed that her vision was becoming "dim." Within a year she could not see to read. She kept the window shades down all day because the light hurt her eyes. For four months before she came to the clinic the pain in her eyes was so intense that she cried most of the time. She was unable to take care of her house or herself, so her granddaughter came to keep house for her.

When we first saw her, she held a cloth over her eyes which, she explained, kept the light out of her eyes. "My eyes feel like they is burned out with red hot poker," she cried over and over again. It took a long time for us to gain her cooperation enough to allow us to examine her eyes. Exuding from both eyes was a mucopurulent discharge from which smears and cultures showed hemolytic staphylococcus and streptococcus and xerosis bacilli. The conjunctivae of both eyes were fiery red, and there was pronounced edema of both bulbar conjunctivae. Severe destruction of the corneas of both eyes, more severe on the left than on the right, was striking (Fig. 5). The lesions were so advanced that some observers thought she had "pyophthalmitis" and not a vitamin deficiency.

A complete physical examination showed that she was obese and had hypertensive heart disease. Her blood pressure was 180/110. The left cardiac border extended to the mid-axillary line.

Soon after the physical examination was finished she was given 15 mg. of riboflavin intravenously. Sixteen hours later she volunteered that her eyes were much less painful, and



Fig. 2.—Note inflammation of conjunctivae of both eyes.



Fig. 3.—Note dramatic improvement twenty-one after riboflavin therapy was initiated.



Fig. 5.—Note destruction of cornea, more severe in the left eye than in the right.

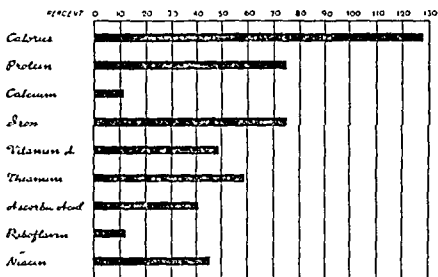


Fig. 6.—Note improvement forty days after riboflavin therapy.



there was a definite decrease in the redness of the conjunctivae. Slit-lamp examination showed that the vessels had decreased in caliber. She was given 15 mg. of riboflavin intravenously daily for two days. By the end of this time much of the redness of the conjunctivae had faded, and there was still further decrease in the caliber of the vessels and in the size of the ulcers in both corneas. She stated that she had scarcely any pain in her eyes. Dr. K. Ascher, who saw the patient at this time, agreed in general with our observations and noted contraction of the vessels in the new scar tissue. She was discharged after ten days in the hospital.

PERCENTAGE OF RECOMMENDED ALLOWANCES OF NUTRIENTS<sup>1</sup>  
SUPPLIED BY DIET OF L. N.<sup>(2)</sup>



<sup>1</sup> Recommended by Council on Food and Nutrition, National Research Council.  
<sup>2</sup> Average daily intake of nutrients based on food intake for one week in March, June, Sept and Dec. 1943.

Fig. 4.

For the next two months her granddaughter brought her to the clinic weekly for observation (see Fig. 6, forty days after initial riboflavin therapy). At the end of this time all the redness of her eyes had disappeared and she could see well enough to come to the clinic alone. She went to visit her daughter in another town and did not come to the clinic again for six months. Her daughter was unable to provide her with a better diet than she had had at home. For five months she had no recurrence of the eye symptoms; then she developed photophobia and lacrimation which grew steadily worse. A month after the onset of these symptoms she returned to the clinic seeking treatment.

Examination of her eyes showed that the vessels of the conjunctivae and corneas were again dilated, and there was beginning ulceration in the corneas of both eyes. There was, however, much less inflammation and purulent exudate than when we had first seen her. Bacteriologic examination showed the same organisms as before. The administration of 15 mg. of riboflavin intravenously was again followed by dramatic improvement. This therapy was continued for ten days, and at the end of this time she could see well enough to read.

Although she was advised to include in her diet daily one quart of milk, a serving of lean meat, two eggs, and two large servings of green vegetables, she was unable to buy these foods and continued to eat a diet grossly inadequate in riboflavin. For the following two years she came to the clinic frequently for observation and treatment. During this time she had six recurrences of the ocular symptoms. Each time the symptoms recurred in the same sequence. The deficiency was never allowed to progress to the place where ulceration recurred. At the time of the first five recurrences she was given 15 mg. of riboflavin intravenously. The sixth time, 15 mg. was administered orally. Eight months ago she began getting an old-age pension and since then her diet has been adequate and she has had no recurrence of symptoms.

This patient's general health and strength are greatly improved despite the fact that she has organic heart disease. At the present time her blood pressure is 168/94. The corneas of both eyes are badly scarred and her vision is not perfect. Nevertheless, she can see well enough to read, to do her own housework, and to travel around town alone, whereas before treatment she had had to be led to the clinic.

This case is illustrative of a patient who had eaten a liberal and varied but especially high carbohydrate diet until she was 62 years of age, when illness



forced her to give up her job and she could no longer buy protective foods. As a result, her diet became not only unbalanced, but also grossly deficient in riboflavin. After eating such a diet for six years, she developed photophobia and lachrymation, severe conjunctivitis, and corneal ulceration of both eyes. Her eyes improved after intravenous riboflavin therapy, but large areas of scar tissue remained. She was unable to obtain a job and therefore could not buy proper food. In two years she had six recurrences of the ocular symptoms. Each recurrence was successfully treated with riboflavin before further irreparable damage to her eyes could develop. Then an old-age pension became available, which has made it possible for her to improve her diet, and she has had no recurrence of symptoms.

Included in this study were three cases of strabismus in which the part of the eye most exposed to light was the part most severely affected, showing that direct light had an effect. The response to treatment, however, was just as good as it was in the other patients.

#### DISCUSSION

During the past six years numerous investigators have described cheilosis or *pérleche* and certain ocular manifestations as deficiency disease syndromes and have attributed them to deficiency of riboflavin in the diet. It is unfortunate that some physicians have regarded these manifestations as pathognomonic of riboflavin deficiency. As these lesions often disappear without any known specific changes having been made, it is difficult to assess the value of any substance in relieving them. Rest in bed or relief from insomnia often are followed by a decrease in the size or the disappearance of such lesions. When other diseases are present, the remedies directed toward treating these diseases often improve the general condition of the patient and the cheilotic lesions and ocular symptoms decrease or disappear. When pellagra is severe enough, the general nutritive state of the patient is often greatly impaired and cheilosis and ocular symptoms are frequently present. It is not uncommon to see general improvement in the nutritional status of the patient with pellagra and a disappearance of cheilosis and ocular lesions following only niacin therapy. Unless the diet becomes adequate, however, these lesions tend to return eventually, even when the patient is taking niacin. In this clinic we have seen cheilosis and ocular lesions identical in appearance to those described in this paper which did not disappear even after the patients had received from 2 to 10 Gm. of riboflavin over varying periods of time. These observations have led us to develop the working hypothesis that cheilosis is a clinical term denoting a lesion which may or may not be relieved by riboflavin therapy. It may be relieved by a number of agents that tend to increase the general health of the patient. In some instances it can be relieved by riboflavin alone. In regard to the ocular lesions we take the same point of view. To us it seems that there are certain fundamental anatomical structures involved in each case and that a number of different factors can predispose toward, or produce, such lesions.

In recent years much healthy skepticism has arisen in regard to the relationship of riboflavin deficiency to ocular lesions and cheilosis. This skepticism is chiefly due to the increasing number of papers in the medical literature in which it is reported that healthy subjects given a diet deficient in riboflavin for varying periods of time failed to develop such lesions. It has not been sufficiently stressed that riboflavin deficiency, if it exists as such, probably is the result of very long-continued subsistence on a diet low in riboflavin. Such diets

are common among persons living in some of the southern areas of the United States. This may explain why we see so many persons in our clinic who have cheilosis and ocular lesions which respond to riboflavin therapy. It also is conceivable that, with what has been called riboflavin deficiency, there may be associated, but as yet unidentified, lesions and that the cheilosis and ocular manifestations are only a part of the syndrome.

In many persons we have seen cheilosis without ocular involvement or ocular involvement without cheilosis. In many others we have seen involvement of only one eye or only one angle of the mouth. The degree of storage of riboflavin and other nutrients in the tissues of the body may play a prominent role in the appearance of these lesions. Year after year we hear these undernourished persons complain periodically of weakness, tiredness, irritability, and other vague symptoms; and in trying to arrange these symptoms into some sort of pattern, one is forced to the conclusion that the body can mobilize vitamins and transfer them from one tissue to another. All our studies on the riboflavin content of the blood tend to indicate that there is little or no fall in the concentration of riboflavin even in the presence of severe lesions. Nevertheless, that there is a tendency for the body to hold riboflavin is indicated by the fact that when we have given persons a diet deficient in riboflavin, the amount excreted in the urine decreases very rapidly and soon falls to normal.

The amount of riboflavin which is produced by intestinal bacterial synthesis may be very great. Naturally the type of bacterial flora concerned and the type of diet is very important. Although we have tried to discover whether or not the body can take riboflavin from the viable bacteria, we have little significant evidence either for or against such an hypothesis. Certainly, the flora of the intestinal tract changes in the later stages of deficiency diseases. It may well be that the intestinal tract, or the flora of the intestinal tract, is altered by deficiency disease so that persons with these diseases may absorb less riboflavin than do persons in good health. We must admit that, at this time, riboflavin deficiency is little understood. Nevertheless, the patients in this study had been ingesting a diet inadequate in riboflavin and they developed lesions which responded to riboflavin therapy. Irrespective of the mechanism, physiology, bacteriology, or biochemistry concerned, it is important for the practicing physician to know that such symptoms may arise and in many instances respond to the administration of riboflavin. At the present time we can do little more than recommend that the nutritional status of all patients with ocular lesions or cheilosis be carefully studied and that riboflavin be administered as a therapeutic test.

#### SUMMARY AND CONCLUSIONS

Three hundred patients who developed ocular disease after subsisting on diets deficient in riboflavin were selected and treated for brief periods of time with intravenous injections of riboflavin. There was a prompt elevation in the concentration of riboflavin in the blood and urine. This elevation was rarely sustained for more than a few hours. Within forty-eight hours there was some subjective improvement in all patients, perhaps because they were carefully selected in order to be certain that they had photophobia and were eating a diet low in riboflavin. The degree of improvement volunteered in 80 per cent of the cases was truly remarkable. Within this period there was observed a diminution in the caliber of the dilated vessels in the eye and a striking decrease in the photophobia and corneal ulceration. Accompanying this improve-

ment was a decrease in the number of hemolytic staphylococci, streptococci, and xerosis bacilli in the exudate from the eyes. Despite the fact that many of the patients included in this study have irreparable eye damage, they are relieved of pain and their vision is improved. Seventy-two per cent of these patients have been able to return to work after months or years of idleness. It is possible, of course, that some of these patients may have severe recurrences and lose their positions. Others may improve slowly and obtain work. We have not followed this group of patients long enough to be certain. These should be regarded only as tentative figures. In fact, we are hesitant about including them at all.

Most of the 300 patients continued to eat their usual inadequate diets with the result that 251 have had recurrences, the first recurrence appearing within from one week to two years after the initial period of therapy. The number of recurrences varied a great deal; a few of the patients have had only one recurrence, and one has had seventeen over a period of two years. In forty-nine patients there has been no evidence of clinical recurrence as yet, despite the fact that their diets have not improved. In all recurrences the symptoms tended to evolve in sequential order in the same patient, although the variety and severity of symptoms and the order of their appearance varied widely from patient to patient.

The observations reported in this paper and similar observations on other persons with ocular manifestations of riboflavin deficiency who have been treated with riboflavin administered either orally or intravenously have suggested four important concepts to the authors: The first is that the ocular manifestations of riboflavin deficiency are common in areas in which deficiency diseases are endemic; that they may occur in either sex and probably at any age (the ages of the patients in the series reported in this study varied from 8 to 92 years); that they may be unilateral or bilateral and may occur with or without cheilosis; and that they may or may not be associated with clinical evidence of other deficiency syndromes. The second is that the physician must administer riboflavin either orally or intravenously, not only through the most interesting part of the disease, namely, during the acute manifestations, but that he must continue this therapy until he is certain that the factors predisposing to riboflavin deficiency have been eliminated. The third is that the patient is likely to have recurrences of the ocular symptoms unless the physician instructs him to eat a diet that includes liberal amounts of lean meat, milk, green vegetables, and other protective foods and unless the patient not only understands but also follows these instructions. The fourth is that the physician who does not have a clear picture of the predisposing and precipitating factors in every case will find it more difficult to treat the patient successfully than the physician who has determined these factors and, insofar as possible, eliminated them.

Until more precise methods are available for assessing the degree of riboflavin deficiency in each patient, there is bound to be some controversy and confusion. Meanwhile it is our hope that these therapeutic concepts may be of some practical value.

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## THE EFFECT OF BARIUM CHLORIDE ON THE HEART

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IN THE course of investigating the action of certain drugs on various fundamental properties of cardiac muscle, observations have been made on the influence of barium chloride on the turtle heart. Part of the present interest in this drug was aroused by statements in textbooks of pharmacology that barium has a digitalis-like action (Sollmann, Goodman, and Gilman). In an earlier paper<sup>3</sup> in which a study of the action of digitalis was reported, the methods herein used were described in detail, and it was shown that the most constant action of digitalis is to shorten the refractory period of heart muscle. There has been quite general agreement that barium does increase the rate of beating, especially when rhythmicity is low, an action opposite to that of digitalis. This property led to the clinical use of barium for patients suffering from a slow idioventricular rhythm.

The effect on rate, contractility, and tonus was observed with whole auricles and ventricular strips suspended in a bath of Ringer's solution and the beat was recorded on a smoked drum. Observations were made on six auricles and

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seven ventricles. In most instances the same tissue was tested on two or three successive days. A concentration of barium chloride 1:100,000 did not influence rate, and in some experiments 1:50,000 produced little or no change, but usually concentrations of 1:50,000 and 1:33,000 were effective. For the auricle the effect on rate appeared to depend on the initial rate of beating. In three experiments slight slowing occurred with the first trial, but when the same tissue was observed on the following day and rhythmicity was lower, the same concentration caused an increase in rate; one of these preparations which had an initial rate of 36 per minute was slowed to 32 by a concentration of 1:50,000; twenty-four hours later when the rate of beating was 26, it was raised to 38 by the same amount of the drug.

The effect on the rate of spontaneously beating strips of ventricle was similar to that for the whole auricle, but in general it may be said that the higher the initial rhythmicity, the less the barium effect, and in consequence the degree of rate change was usually greater in the ventricular strips than in the auricle. In one ventricular strip a concentration of 1:50,000 produced no change in rate; 1:33,000 raised the rate from 12 to 18 per minute, and 1:25,000 caused a further increase to 24 beats per minute. In only one of the seven ventricles did slowing occur, when the rate fell from 21 to 18 per minute with a concentration of 1:33,000; the next day the same concentration raised the rate from 15 to 34. However, the more marked action of barium seen in the older tissue was not always to be related to rate alone; in one experiment the fresh strip beat at 16 per minute and the rate was increased to 22 by 1:50,000 barium chloride; twenty-four hours later the same concentration raised the rate from 16 to 28. No satisfactory explanation can be offered for the more pronounced action of barium that was seen on the second and third days. The bath was always changed once or twice after each experiment, but no attempt was made to wash the tissue free from barium chloride. Moreover, as will be pointed out later, no similar behavior was observed for contractility.

When auricular tonus waves were initially present, they persisted after the introduction of barium into the bath, and at times that phenomenon was heightened. In one auricular preparation, tonus waves first appeared following barium, and that event was again observed on the second day. Tonus, indicated by diastolic length, often increased both in auricular and ventricular preparations; relaxation due to the drug was never seen.

The power of barium to produce extrasystoles and abnormal rhythms in the mammalian heart, which has been stressed by Rothberger and Winterberg<sup>4</sup> and Smith, Winkler, and Hoff<sup>5</sup> and which was found disturbing in clinical usage by McMillan and Wolferth,<sup>6</sup> was seldom exhibited in the spontaneously beating turtle heart. In no auricular preparation and in only one ventricular strip did extrasystoles first appear after barium. But when ventricular strips were rhythmically driven by condenser shocks following the addition of barium, there commonly appeared extrasystoles and rapid spontaneous rhythms whose rates were often faster than obtainable by driving before barium was used.

The primary effect of barium on contractility could not be satisfactorily studied because of difficulty in maintaining the necessary constant rate. In such preparations of cold-blooded heart the size of the beat decreases when the rate rises. Those observations that were made indicated a somewhat inconstant effect. At times there occurred a slight increase in strength of beat, even for a faster rate, but the late effect, from twenty to thirty minutes after

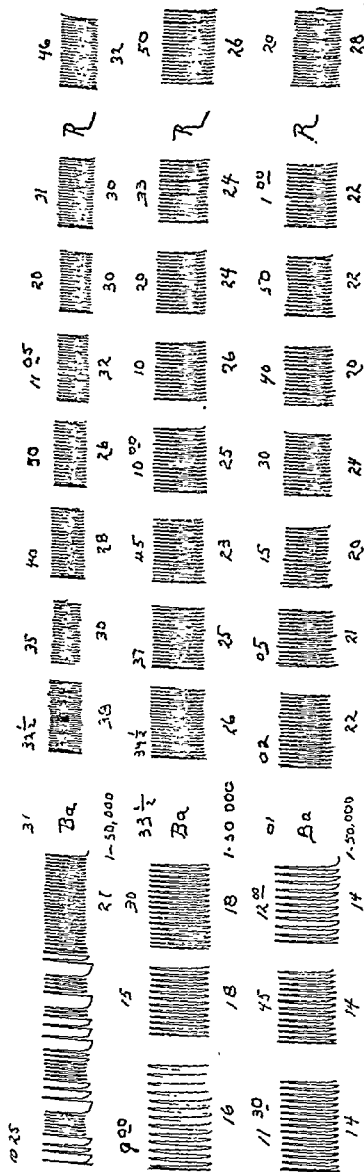


Fig. 1.—To illustrate the effect of barium chloride on the same strip of ventricular muscle on three successive days. Time above kymogram; rate below.

the addition of the drug, was usually a definite decline in beat. In one of the auricles a decrease in beat was caused by a concentration of 1:100,000, which did not effect the rate. The ventricular strips usually showed a decrease in strength of beat independent of rate change. When the bath containing barium was replaced by fresh Ringer's solution, only partial recovery occurred within a short time. However, barium did not cause irreversible impairment of contractility, for when the tissue was tested after twenty-four or more hours, a good beat was recorded. This is well shown in Fig. 1, which represents a ventricular strip that was subjected to barium on three successive days; the tissue was kept in the cold room when not under observation, and the lever tension was constant throughout the period. The greater effect of barium on succeeding days that has been described in connection with its enhancement of rhythmicity was not observed on contractility.

Measurement of the refractory period and of conductivity by electrograms from ventricular strips was rendered difficult because of inability to maintain the tissue at a constant rate of rhythmic stimulation before and after barium. For example, in one experiment the muscle would not follow the usual driving rate of 24 per minute; a rate of 14 could be maintained, but after barium 1:100,000, a spontaneous rhythm of approximately 15 developed, and when barium 1:50,000 was added, the rate rose to 35.7 per minute. According to Sollmann,<sup>1</sup> barium increases excitability of heart muscle. In the few experiments for which satisfactory measurements could be made, the state of excitation, as indicated by the refractory period and measured by the duration of the Q-T interval, was little altered. Change, when present, was usually toward a shortened interval, but the maximum shortening observed was less than 5 per cent (Table I).

TABLE I. EFFECT OF BARIUM ON REFRACTORY PERIOD AND CONDUCTION

EXPERIMENT	D. R.	S. R.	Q-T	Q-Q	BARIUM	Q-T	Q-Q	S. R.
A	24.2	8.5	1.26	.04	1:50,000	1.36	.04	27.0
B	24.2	16.7	1.4	.30	1:100,000	1.34	.28	23.1
	24.2		1.4	.20	1:50,000	1.36	.27	23.1
C	24.2		1.8	.38	1:100,000	1.8	.42	
	24.2		1.78	.42	1:50,000		.32	
D	24.2		1.48	.32	1:100,000	1.44	.32	
	24.2		1.40	.34	1:50,000	1.34	.36	
E	24.2		0.92	.24	1:100,000	1.0	.20	
	24.2		1.0	.05	1:50,000	1.0	.06	

D. R., Rate of rhythmic stimulation.

S. R., Rate of spontaneous beating.

Q-T, Refractory period.

Q-Q, Conduction time between receiving electrodes.

In the normally beating dog heart Rothberger and Winterberg<sup>4</sup> found barium to have no appreciable effect on A-V conduction. Megibow and Katz,<sup>7</sup> who maintained auricular fibrillation in the dog heart, reported that barium chloride enhanced A-V conduction and that the effect was marked and prolonged. In the strips of turtle ventricle, with one exception, conduction effects were slight, and the direction was not constant (Table I). In these experiments the effect on both refractory period and conductivity was minimal.

#### SUMMARY

The effect of barium chloride on the whole auricle and on strips of ventricle of the turtle heart has been studied. The principal action of the drug was

to increase rhythmicity. This effect was more marked when the rate of beating was relatively low, and also when the tissue had been removed from the animal for twenty-four or more hours. Contractility was usually depressed. The influence on excitability, measured by the Q-T of the electrogram, and on muscle fiber conduction was slight.

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## ON THE USE OF THEOPHYLLINE AMINOISOBUTANOL IN ANGINA PECTORIS

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## INTRODUCTION

THE use of theophylline and its derivatives in the treatment of coronary disease has persisted for many years in spite of lack of conclusive evidence regarding its efficacy. There is no doubt that, given intravenously, it has a marked effect upon the myocardium and upon the coronary circulation. It has further been assumed that a similar effect could be obtained by oral administration. In recent years, Smith and co-workers<sup>1</sup> and Riseman<sup>2</sup> have defended the view that theophylline given routinely by mouth helps in the treatment of angina pectoris. However, Smith's data were not adequately controlled and Riseman's covered a wide field of drugs. His results were given in general terms without details. In only one chart is plotted the effect of theobromine upon the exercise tolerance of one patient. The conclusions of these authors have to some extent been based upon a subjective feeling of improvement in the patients. No attempt has been made to discount the stimulating effect of theophylline upon the central nervous system. None of these observations equal in care those published by Evans and Hoyle<sup>3</sup> in 1934. The technique of these authors seemed to us the best available and we have employed it in the present study. Evans and Hoyle failed to demonstrate a decrease of the number or severity of the attacks of angina pectoris in patients

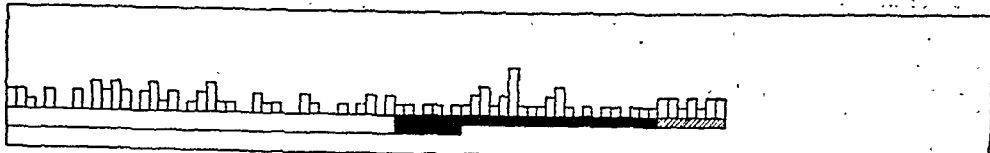
From the Department of Medicine, Washington University School of Medicine, and the St. Louis City Hospital, Max C. Starkloff Memorial.

This study was supported by a grant from the Wm. S. Merrell Co., Cincinnati, Ohio.

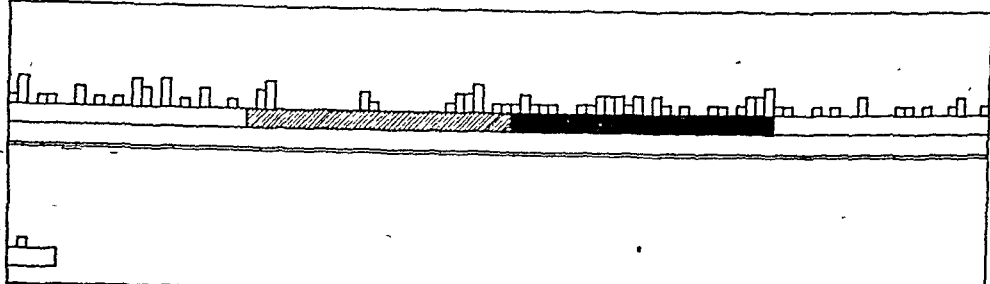
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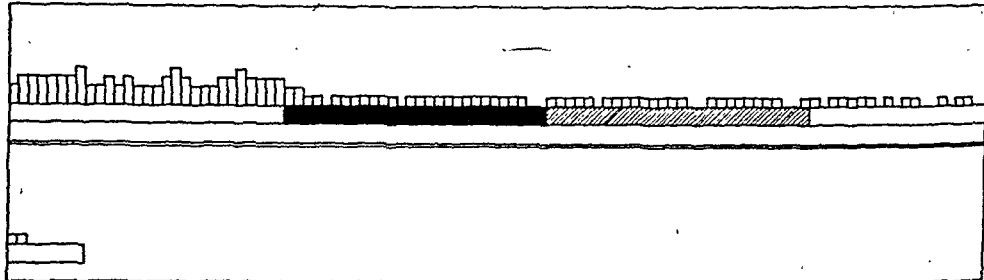
CASE #1



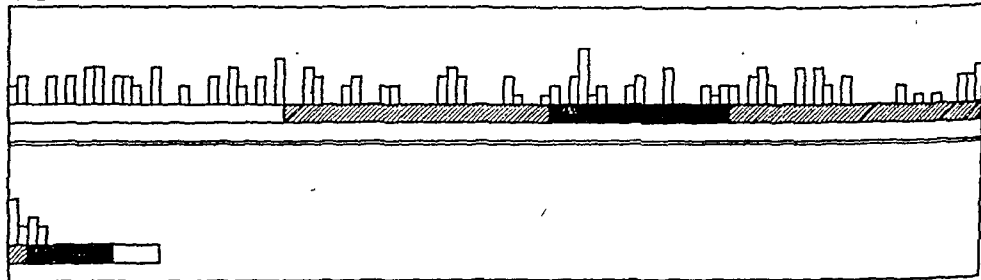
CASE #2



CASE #3



CASE #4



CASE #5

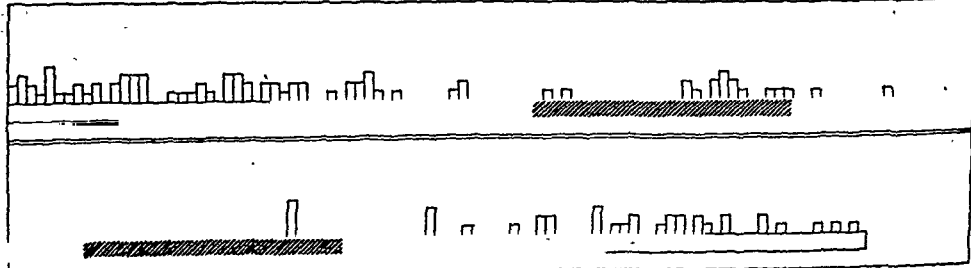
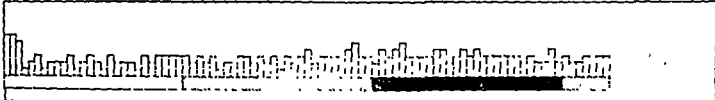
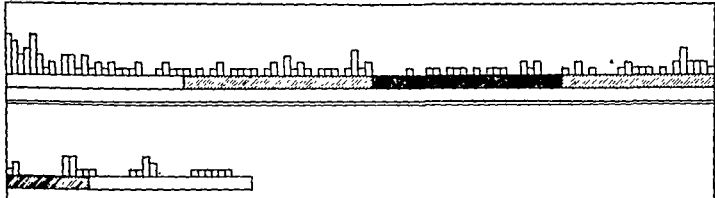


Fig. 1.—Each column represents one day; the height represents the number of attacks recorded, each square indicating one attack. The base band indicates medication: white, no medication; black, theophylline aminolsobutanol; gray, placebo.

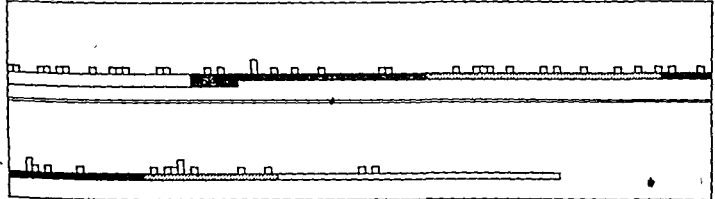
CASE #6



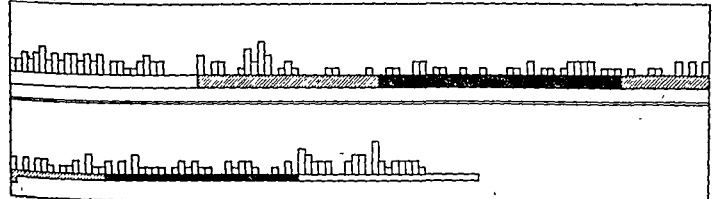
CASE #7



CASE #8



CASE #9



CASE #10

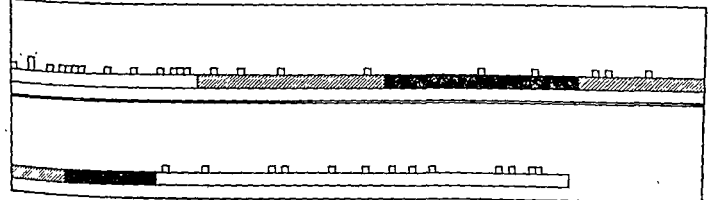


Fig. 1 (Cont'd).—See opposite page for legend.

taking theophylline. In 1943, Boyer<sup>4</sup> reviewed the literature on the subject and agreed with Evans and Hoyle that, given orally, theophylline is without effect upon the coronary circulation.

It had been suggested that the reason for this failure to obtain demonstrable effects by oral administration was insufficient dosage, for larger doses cause gastric irritation. Enteric coating does not solve the problem, for tablets so treated may pass undissolved through the intestine. Attempts have been made to produce theophylline preparations which do not upset the stomach. Theophylline aminoisobutanol\* is one such preparation. It contains 67 per cent theophylline.

Theophylline aminoisobutanol was given by mouth by Dr. Drew Petersen, on our service, in doses up to 6 gr. three times a day. While good tolerance was not universally observed, the drug was tolerated well enough to be used for an extended study of its effect on ambulatory patients with angina pectoris.

Because theophylline continues to be, more or less wholeheartedly, recommended for oral administration in coronary disease, because it is so widely used in actual practice, and because it was thought that perhaps the Wm. S. Merrell Company had overcome the principal objection to its oral use, it was decided once more to test theophylline in general and specifically the new derivative, theophylline aminoisobutanol.

#### TECHNIQUE

The technique used by Evans and Hoyle was closely followed. The patients kept diaries of their attacks and the time of observation was divided into periods of four weeks each. After a preliminary period of observation, periods of taking theophylline aminoisobutanol and periods when the patients received a similar but inert tablet followed in irregular sequence.

Sixteen patients with angina pectoris were selected for this observation. Ten of these continued under observation for from twelve to twenty-four weeks. Only in these were the findings considered sufficiently complete to allow conclusions. Nine of them had arteriosclerotic heart disease and one had syphilitic heart disease, which probably caused his angina.

The diagnosis of angina pectoris was established by a carefully taken history, emphasis being placed upon the relation of the pain to effort, emotion, meals, or weather and on the effect of glyceryl trinitrite. If a myocardial infarction had occurred within six months, the patient was not accepted. The patient was given a complete physical examination before he was accepted and electrocardiograms were taken repeatedly during the period of observation.

The patients were seen weekly by one of us (F. S.). They recorded their attacks on a special form and graded them 1 to 4 on the basis of severity and duration. They also kept count of the number of glyceryl trinitrite tablets used per week and, finally, they were asked their subjective impressions of the week as a whole, especially regarding factors which might influence their attacks (emotional factors, unusual efforts, intercurrent illnesses, changes of weather).

During the entire period the patients were instructed to use glyceryl trinitrite as needed and a few who had previously taken phenobarbital routinely continued to do so. After four (in one case three) weeks of observation, some of the patients were given theophylline aminoisobutanol, .18 Gm. three times a

\*Produced by W. S. Merrell Co.

day; the others received as many inert tablets of similar appearance. Every four weeks the drugs were changed without the patient's knowledge so that those who had received the drug were now given the inert substitute and vice versa. Three patients who complained of nervousness and nausea (in one case while the placebo was being given) had their dosage reduced by one-half. Finally, in five of the cases, the test periods were followed by additional weeks of observation, during which the patients received neither theophylline aminoisobutanol nor its inert substitute.

Despite all precautions the evaluation of our results proved very difficult. As is well known, angina pectoris is subject to considerable spontaneous variations and is easily influenced by effort, emotion, and other factors, the exact effect of which it is difficult to define in any given case. Furthermore, many patients with angina pectoris are easily influenced and the mere increased attention which they received made them feel better. Some were overanxious to report improvement and would ignore minor attacks, such as they had formerly recorded. All of these means of investigation, therefore, proved unreliable. We found that even the amount of pain which induced the patients to take glyceryl trinitrite tablets varied so much that the number of such tablets taken proved no reliable guide to the effect of our theophylline therapy.

We finally concluded that the number of recorded attacks of angina pectoris was the best indication of such effect. These data were so arranged as to bring out any contrast between the periods when active and inactive drugs were administered. On squared paper a base band was drawn; the space was white when no drug was given, black when theophylline aminoisobutanol was given, and gray when the inert substitute was given. All days of observations were indicated by spaces on this band. On each day one definite attack was indicated by a square, the number of squares indicating the number of attacks on any given day. Such graphic representations of the ten cases which were studied for from eleven to twenty-four weeks are reproduced in Fig. 1.

#### CONCLUSIONS

It will readily be seen that there is no significant absence of attacks of angina pectoris during the periods when the patients received theophylline aminoisobutanol as contrasted with the periods when they received the inert substance or no drug at all. It is concluded that theophylline in general, and specifically theophylline aminoisobutanol, exerts no demonstrable effect upon the incidence of attacks in patients with angina pectoris.

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## DIMINUTION OF EPINEPHRINE SENSITIVITY OF THE NORMAL HUMAN HEART THROUGH THIOURACIL

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THE outstanding role played by excessively secreted epinephrine, sympathin, and related catechol compounds as myocardium-anoxiating and myocardium-damaging agents in the pathogenesis of various forms of heart disease, such as angina pectoris, myocardial degeneration, uremic heart, and sudden cardiac death,<sup>1a-d</sup> makes it desirable to protect the heart muscle against the continually or paroxysmally exerted toxic chemical effects of the sympathomimetic amines named. This can be attempted either by reducing exaggerated adrenal medullary secretion through x-ray irradiation of the adrenal region<sup>2</sup> or by removal of an adrenal medullary tumor or, on the other hand, by artificial diminution of the sensitivity of the heart to epinephrine.

A marked intensification of the stimulating and potentially damaging effects of epinephrine on the heart through the thyroid hormone is a well-established fact.<sup>1e, 3-9</sup> Thyroidectomy produces the opposite situation: the cardiac effects of epinephrine are diminished.<sup>6, 10-12</sup> The beneficial results of thyroidectomy in angina pectoris and heart failure<sup>13, 14</sup> are, accordingly, essentially attributed to the elimination of the thyroid hormone as a factor which sensitizes the heart muscle to the anoxiating action of secreted epinephrine and sympathin.<sup>1a, 11, 12</sup>

It was to be anticipated that thiouracil treatment, by suppressing thyroid secretion,<sup>15</sup> would yield analogous results, and both experimental and clinical observations corroborate this assumption. In rats the resistance of the heart muscle to injected toxic doses of epinephrine and to excessive myocardial epinephrine accumulation was found to be markedly increased after administration of thiouracil,<sup>16</sup> and striking therapeutic results were obtained in patients with angina pectoris through treatment with thiouracil, while the symptoms reappeared when placebos were substituted for the drug without the patient's knowledge.<sup>17</sup>

The following observations were carried out in order to ascertain the diminishing effect of thiouracil on the epinephrine sensitivity of the normal human heart.

### METHOD

Ten physically healthy young male patients of the Vermont State Hospital were subjected to thiouracil medication for three months (daily total dose, 0.4 Gm. in two portions\*). Before and at the end of the medication period the following tests were obtained: basal metabolic rate, blood pressure, pulse rate, and electrocardiogram at rest; electrocardiogram immediately after exercise (36 times hopping on one foot); blood pressure and pulse rate after subcutaneous

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\*The drug was supplied in generous quantities by Lederle Laboratories, Inc., Pearl River, N. Y.

injection of from 0.5 to 1.0 mg. of epinephrine during thirty minutes, measured at six-minute intervals; electrocardiogram taken twelve and thirty minutes after injection.

The psychiatric diagnoses of the test subjects were dementia praecox and psychoses with mental deficiency. While the patients were fairly cooperative as a whole, it was not possible in some of them to establish ideal standard conditions for basal metabolism determination and the readings, therefore, must be regarded with caution.

No unpleasant side reactions were complained of after epinephrine injection; there was some pallor but no trembling, no intense palpitation, and no precordial distress. The thiouracil tablets were well tolerated. Weekly leucocyte counts showed normal values without exception. There developed no clinical features of hypothyroidism.

### RESULTS

**Basal Metabolic Rate.**—Marked decreases below the zero line occurred in only two patients (Cases 7 and 9). In four other patients there were minor decreases. The increases in two patients (Cases 3 and 10) may be explained by the distinctly changed mental state of these subjects at the times of the determinations.

**Blood Pressure and Pulse Rate.**—Changes of the blood pressure level observed after thiouracil medication were rather irregular (Tables I and II), but there was a slight decrease of the average systolic and diastolic pressure. The same applies to the pulse rate at rest.

TABLE I

CASE	AGE (YR.)	B.M.R.		BLOOD PRESSURE AT REST				PULSE RATE AT REST		AVERAGE* REACTION TO EPINEPHRINE INJECTION							
				SYSTOLIC		DIASTOLIC				BLOOD PRESSURE				PULSE RATE			
										SYSTOLIC		DIASTOLIC					
		BEFORE THIOU- RACIL	AFTER THIOU- RACIL	BE- FORE THIOU- RACIL	AFTER THIOU- RACIL	BE- FORE THIOU- RACIL	AFTER THIOU- RACIL	BE- FORE THIOU- RACIL	AFTER THIOU- RACIL	BE- FORE THIOU- RACIL	AFTER THIOU- RACIL	BE- FORE THIOU- RACIL	AFTER THIOU- RACIL	BE- FORE THIOU- RACIL	AFTER THIOU- RACIL		
1	27	+ 3%	+12%	122	112	84	64	76	80	+11	+21	-21	-15	+10	+ 1		
2	38	- 1%	- 5%	122	120	68	66	68	64	+ 7	+ 9	- 5	- 8	+10	+ 9		
3	32	-14%	+ 5%†	130	126	68	78	72	54	+32	+23	-48	-33	+6	- 2		
4	37	+20%	+14%	130	138	72	80	76	76	- 0	+ 5	- 2	-15	+ 4	- 3		
5	25	+12%	± 0%	116	106	82	58	76	78	+ 4	+ 4	-12	-15	+10	- 4		
6	22	+ 9%	+ 5%	116	106	68	68	72	64	+10	+10	- 9	-10	+12	- 7		
7	22	- 3%	-10%	128	112	78	86	96	92	+ 4	± 0	-28	+ 3	+ 9	- 3		
8	34	+ 6%	+ 6%	134	140	90	86	98	90	+ 5	+ 6	- 9	-16	+ 5	-10		
9	23	+ 1%	-23%	148	156	80	90	88	88	+ 6	+15	-22	-23	+ 1	+10		
10	23	-18%‡	- 4%‡	108	98	68	32	66	52	+ 9	+ 5	-12	+ 3	+ 4	+ 2		

\*Average of five readings taken during thirty minutes after injection of 0.5 mg. of epinephrine (Cases 5, 6, and 10) and of 1 mg. in the other seven patients.

†Tense and restless.

‡Depressed and mute at time of first determination; rather excited at the time of the second determination.

Also, the reactions of the blood pressure to epinephrine injection were only slightly and uncharacteristically altered after thiouracil medication. However, the epinephrine-induced acceleration of the heart was diminished throughout after thiouracil with only one exception, and in six patients the pulse rate was even decreased after epinephrine injection following thiouracil treatment.

**Electrocardiogram.**—At the end of the thiouracil period there were a few rather irregular changes of the resting electrocardiogram as compared with the original details. In two patients there was an inversion of the T wave (T<sub>1</sub> in

TABLE II. AVERAGE VALUES OF TEN PATIENTS BEFORE AND AFTER THREE MONTHS OF TREATMENT WITH THIOURACIL (DAILY, 0.4 GM.)

	BEFORE THIOURACIL	AFTER THIOURACIL	DIFFERENCE
Basal metabolic rate	+1.5%	-1.0%	-2.5%
Body weight	152 lb.	152 lb.	±0 lb.
Systolic blood pressure	125 mm.	121 mm.	-4 mm.
Diastolic blood pressure	76 mm.	71 mm.	-5 mm.
Pulse pressure	49 mm.	50 mm.	+1 mm.
Pulse rate per minute	79 beats	74 beats	-5 beats
<i>Response to subcutaneous injection of epinephrine</i>			
Elevation of systolic blood pressure*	8 mm.	10 mm.	+2 mm.
Fall of diastolic blood pressure*	17 mm.	13 mm.	-4 mm.
Increase of pulse pressure*	26 mm.	24 mm.	-2 mm.
Change of pulse rate	+7 beats	-1 beat	-8 beats

\*Total average of the individual averages of five readings taken at six-minute intervals during thirty minutes following injection in each patient.

Case 7,  $T_2$  and  $T_3$  in Case 8).  $T_4$  was slightly lowered in eight patients; the T waves in other leads were slightly lowered or slightly increased in a minority of instances. No low voltage of the myxedematous type was seen in any patient.

Following physical exercise only four persons (Cases 5, 7, 9, and 10) showed appreciable alterations of the electrocardiogram (lowering of the T wave in one to three leads) prior to thiouracil treatment. After thiouracil treatment these reactions were only slightly modified (Table III), but with an average diminution of the depression of  $T_1$  and  $T_4$ .

TABLE III. DIFFERENCES BETWEEN THE ALTERATIONS OF THE T WAVES DUE TO PHYSICAL EXERCISE BEFORE AND AFTER THIOURACIL (MILLIMETERS)

CASE	$T_1$	$T_2$	$T_3$	$T_4$
5	-1.0	0	-0.5	-5.0
7	-0.5	0	0	+0.5
9	-1.0	0	+0.5	+1.5
10	-0.5	0	0	-1.5
AVERAGE	-0.8	0	0	-1.1

Epinephrine injections before thiouracil were followed by alterations of the T wave in two or more leads in most patients (lowering in twenty-seven instances, increase in nine, and no change in four). After thiouracil treatment these electrocardiographic responses to epinephrine were slightly to markedly weakened in all subjects (Table IV), the depression of the T waves being diminished or entirely abolished. Prethiouracil inversions of  $T_1$ ,  $T_3$ , and  $T_4$ .

TABLE IV. DIFFERENCES BETWEEN THE ALTERATIONS OF THE T WAVES DUE TO EPINEPHRINE (TWELVE AND THIRTY MINUTES AFTER INJECTION) BEFORE AND AFTER THIOURACIL (MILLIMETERS)

CASE	$T_1$		$T_2$		$T_3$		$T_4$	
	12 MIN.	30 MIN.	12 MIN.	30 MIN.	12 MIN.	30 MIN.	12 MIN.	30 MIN.
1	-1.0	-1.0	-1.0	-0.5	0	0	-4.0	-3.5
2	0	0	+0.5	+0.5	-0.5	-0.5	-1.5	-1.5
3	-1.0	-1.0	-1.0	-1.5	-0.5	-0.5	-1.5	-3.0
4	0	-0.5	-0.5	-0.5	-0.5	0	-1.0	-3.5
5	-1.5	-2.0	+0.5	0	-0.5	-1.0	-4.0	-4.0
6	-1.5	-1.0	-4.0	-4.5	-7.5	-7.5	-2.5	-0.5
7	-1.0	-1.0	-0.5	0	-0.5	-0.5	-2.5	-1.0
8	+0.5	0	-0.5	0	0	-0.5	-1.0	-0.5
9	-0.5	-0.5	0	0	0	0	-2.0	-1.5
10	-1.0	-0.5	-0.5	-0.5	-1.0	-1.0	-0.5	-0.5
Average	-0.70	-0.77	-0.70	-0.72	-1.1	-1.2	-2.1	-2.1

due to epinephrine (Cases 6 and 7) were absent after thiouracil treatment. Only in four of seventy-six individual single leads was there a very slight (0.5 mm.) elevation of T waves compared with the corresponding leads before thiouracil. The averages (Table IV) show a diminished response of the T waves to epinephrine in all leads after thiouracil treatment both twelve and thirty minutes after injection.

#### DISCUSSION

The irregular and altogether insignificant changes of the systolic and diastolic blood pressure and of their response to epinephrine injection following inhibition of thyroid function through three months of thiouracil treatment are essentially in agreement with similar results obtained by Riseman, Gilligan, and Blumgart<sup>14</sup> in man after total thyroidectomy.

The resting pulse rate did not show any characteristic alteration after thiouracil, but the epinephrine-induced cardiac acceleration was regularly diminished with only one exception, and in more than half of the patients it was even transformed into a retardation. No clear relationship was observed between this behavior of the heart rate and the degree of response of the basal metabolic rate to thiouracil. A rather wide individual range of the latter has also been noted by other observers<sup>15</sup> in thyrotoxic patients and by me<sup>14</sup> in ten nonthyrotoxic patients with angina pectoris.

According to Riseman and co-workers,<sup>14</sup> the "cardiovascular sensitivity" to epinephrine (not including the electrocardiogram) remains unchanged after thyroidectomy unless the basal metabolic rate falls below -30 per cent. Levels as low as this were not reached in the thiouracil studies discussed herewith. Eppinger and Levine<sup>12</sup> observed a diminution of the blood pressure and pulse reactions to epinephrine soon after thyroidectomy but not regularly. Leblond and Hoff<sup>13</sup> found the heart rate and heart size of thiourea-treated rats diminished. DiPalma and Dreyer<sup>18</sup> did not notice any change of the effect of epinephrine on the intestinal movements of three rats following from one to three weeks of treatment with thiourea. Cardiovascular reactions were not included in these experiments, however. Acute single administrations of thiourea were ineffective regarding the response of blood pressure and pulse rate to epinephrine as one would expect in view of the slowness with which thiourea leads to a hormone depletion of the thyroid gland.

From the foregoing discussion of personal observations with thiouracil it would appear that the epinephrine sensitivity of the heart is much more affected by reduction of the thyroid function than that of the peripheral vascular system. This is demonstrated not only by the almost exceptionless diminution of the heart rate reaction, but also by the weakening or abolition of the electrocardiographic effects of secreted (exercise test) and injected epinephrine after thiouracil in all cases. Numerous observations<sup>14</sup> have shown that the electrocardiogram is a sensitive criterion for the epinephrine-induced myocardial anoxia.

The heart muscle possesses an outstanding tendency to accumulate injected epinephrine.<sup>14</sup> The epinephrine tolerance-decreasing effect of thyroxine as well as the epinephrine tolerance-increasing effect of thiouracil, which renders otherwise inevitably fatal myocardial epinephrine concentrations innocuous,<sup>16</sup> are in keeping with the conception of an indirectly protective effect of thiouracil on the heart through elimination of the epinephrine-potentiating thyroid hormone.



Striking therapeutic results obtained by me<sup>11</sup> in angina pectoris patients with thiouracil therapy and the normalization of the anoxic electrocardiogram in some of these patients were achieved and interpreted on the basis of this same rationale. They seem to contradict the conclusion of Riseman and co-workers<sup>10</sup> that the therapeutic effect of thyroidectomy in angina pectoris is mainly due to the mechanical interruption of sensory impulses from the heart to the central nervous system. On the other hand, they corroborate the opinion of Eppinger and Levine,<sup>12</sup> Shambaugh and Cutler,<sup>11</sup> and Raab<sup>11</sup> that thyroidectomy improves the anginal symptoms by reducing the sensitivity of the heart to secreted epinephrine.

The blood pressure of patients with angina treated with thiouracil remained unchanged. It is not directly involved in the mechanism of angina pectoris.

Neither in patients with angina nor in the physically normal persons examined in this study was there a strict parallelism between the response to thiouracil of the basal metabolic rate on one side and of the cardiac condition on the other. Disappearance of the anginal symptoms followed the basal metabolic rate much more closely than was the case regarding the epinephrine resistance of the electrocardiogram. This apparent divergence may be explained by the questionable accuracy of the basal metabolism readings in some of the mentally abnormal and, in part, slightly tense subjects used for the latter observations. The conclusion may be justified, however, that the fall of the basal metabolic rate is just one manifestation of reduced thyroid activity and that the diminished epinephrine sensitivity of the heart is a separate one, not directly dependent on the degree of reduction of general oxygen consumption of the body. The statement of Leblond and Hoff<sup>17b</sup> that the effect of thyroxine on the heart is a direct one and not the consequence of general metabolic stimulation is in agreement with this view.

#### CONCLUSION

Thiouracil administration for three months to ten physically healthy persons was followed by a significant diminution of the sensitivity of the heart to epinephrine (heart rate, electrocardiogram).

The behavior of the blood pressure was rather uncharacteristic.

Implications of these observations regarding the pathogenesis and treatment of angina pectoris are briefly discussed.

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## FATAL AGRANULOCYTOSIS OCCURRING DURING TREATMENT WITH THIOURACIL

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**T**OXIC reactions during the course of thiouracil therapy have been reported. Their incidence is about 10 per cent.<sup>1</sup> Most of these are mild and disappear when the drug is discontinued or its dose reduced. Unfortunately, cases of agranulocytosis have been observed as a complication, and practically every publication stresses the possibility of its occurrence. Some of these cases have ended fatally. The necessity of continuous clinical and laboratory studies, the repeatedly stressed fatalities, and probably the unavailability of the drug are factors responsible for the extremely cautious attitude toward thiouracil. In our experience a great majority of the profession voices the opinion that "many deaths" have occurred during its experimental trial. Some statements in the literature contribute to this belief, but there is confusion concerning fatalities.

The first case of agranulocytosis occurring during thiouracil therapy was published by Astwood,<sup>2</sup> and although the patient recovered, this case was repeatedly quoted as fatal agranulocytosis.<sup>3, 4</sup> Newcombe and Deane's<sup>5</sup> case, in which the patient recovered, was referred to similarly. In Himsworth's<sup>6</sup> own case of agranulocytosis, the patient's blood count had become elevated (26,000

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white blood cells; 76 per cent neutrophiles) when the patient contracted bronchopneumonia and died. A "third fatal case"<sup>3</sup> was mentioned but details were not given.

Actually, there were no cases of fatal agranulocytosis published in detail before the reports of Kahn and Stock,<sup>6</sup> Ferrer, Spain, and Cathcart,<sup>4</sup> and Gargill and Lesses.<sup>7</sup> We are now reporting a fourth fatal case occurring during treatment with thiouracil albeit this tends to emphasize the fatalities.

#### CASE REPORT

The patient, a 50-year-old widow, was referred for care of hyperthyroidism. The family history revealed that the mother died of a goiter and heart trouble at 54 years of age. One sister had hyperthyroidism. The past history revealed that the patient had had three full-term uneventful pregnancies. At 40 years of age (1934) she developed peritonitis following an abortion and was hospitalized for three months. In 1940 an incisional hernia was repaired. She was hospitalized in February, 1943, for an upper respiratory infection, for which she received approximately 22.0 Gm. of sulfathiazole without incident. Menopausal symptoms began in this year (age, 49) and she was treated with estrogenic substance. This improved a mild neurodermatitis that had proved resistant to various dermatologic therapeutic measures. We are unable to state whether or not this represented some form of allergy. In March, 1944, she weighed 162 pounds, and since she was only 60 inches tall, she sought medical care for reduction in weight. Her physician prescribed amphetamine sulfate. No basal metabolic rate was determined. She lost 16 pounds on this management and in July noticed that she was nervous and that the hot flashes recurred. Friends noted a protrusion of her right eye, for which she consulted her ophthalmologist (Dr. M. H. Cottle), who referred her for care of the hyperthyroidism.

The physical examination revealed a slightly obese woman with a warm moist skin and tachycardia. The thyroid was symmetrically enlarged and smooth. The blood pressure was 150/90 mm. Hg. There was a long midline suprapubic scar and a few lower extremity varicosities. Right unilateral exophthalmos was present.

Laboratory studies revealed plus 43 to plus 85 basal metabolic rates; 132 mg. per cent blood serum cholesterol; normal blood count and urine. She was given 0.6 Gm. thiouracil\* for thirty-one days, 0.4 Gm. daily for fourteen days, 0.2 Gm. for twenty-one days, and 0.1 Gm. for ten days; the total dose was 29.4 Gm. The basal metabolic rate was reduced markedly after six weeks, the pulse rate became slower, and no change was noted in the blood count or weight. The exophthalmos and general condition improved dramatically.

The patient was seen November 13 when her throat was slightly injected and her temperature was 99.6° F. The lungs were clear. Thiouracil was stopped. The throat condition became worse, until on November 15 swelling, marked redness, and a dirty white exudate were observed. The temperature was 103° F. She had become toxic and restless. She was hospitalized and the blood count showed 84 per cent hemoglobin (Haden-Hausser), 4,980,000 red blood cells per cubic millimeter and 2,500 white blood cells. No neutrophiles were found in the film. Mild jaundice was noted. The white count upon successive days was 3,000, 1,200, and 500. The final differential count (consultation by Dr. R. Isaacs) was polymorphonuclears, 4; polymorphonuclears, young, 2; lymphocytes, 59; monocytes, 18; endothelial cells, 10; plasma cells, 7.

The patient died November 19 with a temperature of 106° F. after a deepening coma in spite of receiving, daily, 40 c.c. of pentnucleotide intramuscularly, 6 c.c. yellow bone marrow, 500 mg. ascorbic acid, 100 mg. thiamine chloride, a transfusion of 500 c.c. of whole blood, and 10 units of crude liver extract intramuscularly. She also received a few oral 2 c.c. doses of folic acid and 15,000 units of penicillin every two hours for three days along with continuous oxygen therapy.

The post-mortem examination (performed by Dr. J. P. Simonds) revealed a diffuse large ulceration of the entire pharyngeal wall extending to the pyriform fossae. It was covered by a dirty grayish membrane, the bases being irregular and dark reddish purple in color. The ulceration covered the arytenoepiglottic folds and the internal surface of the epiglottis. The area surrounding the ulcer was swollen.

\*The thiouracil used was supplied by the Upjohn Company, Kalamazoo, Mich.

The thyroid weighed 22.5 grams. There was a circumscribed grayish nodule in the left lobe. Sections showed a fairly well encapsulated mass composed of relatively small acini lined with cuboidal epithelium. The small lumina were empty or were filled with a thin homogeneous coagulated material. The stroma near this nodule was thin and structureless. In other areas there were numerous bands of connective tissue stroma that divided the parenchymatous tissue into irregular masses. The acini varied in size and were lined with tall columnar epithelium, forming invaginations into the larger lumina. Colloid was present in very few of these acini. Areas of lymphocytic infiltration were common.

The adrenals were slightly larger than normal with the cortex approximating 3 mm. in thickness. The outer zone was yellow and the inner zone was brown. Microscopically the adrenals showed a marked congestion of the medulla and the inner portion of the cortex. Lymphoid material was not present in the cortex in a normal quantity.

A section of the bone marrow showed the bony trabeculae to be normal. The marrow appeared cellular; many megakaryocytes and cells of the erythroblastic series were present. A few promyelocytes and myelocytes but no mature granulocytes were noted.

#### DISCUSSION

The connection between chemotherapeutic drugs and agranulocytosis was pointed out by Kracke.<sup>8</sup> With the introduction and wide use of the sulfonamides, the importance of this complication gained new emphasis. The relation between the action of thiourea derivatives and sulfonamides has been noted repeatedly. That thiouracil also may have toxic effects on the bone marrow was learned early in its clinical use. One of the first three patients with hyperthyroidism treated with thiouracil developed agranulocytosis (Astwood<sup>2</sup>). Later experiences differ significantly. McGavack and associates<sup>9</sup> encountered one case of agranulocytosis and three cases of leucopenia among twenty-six patients treated. Astwood<sup>10</sup> more recently reported one case of agranulocytosis and one of leucopenia in a total of sixty-two treated patients. Himsworth<sup>3</sup> reported one instance of agranulocytosis and two of leucopenia among twenty-two patients. Gargill and Lesses<sup>7</sup> observed one fatal case of agranulocytosis and one of leucopenia among forty-four patients. Williams (quoted by Jackson<sup>11</sup>) treated 210 patients and observed agranulocytosis twice. Grollman and Gryte<sup>12</sup> noted four cases of leucopenia developing on the third or fourth day of treatment in eighteen patients. In Rose and McConnell's<sup>13</sup> series of thirty-seven patients two had leucopenia with pharyngitis. Gabrilove and Kert<sup>14</sup> noted leucopenia in one of nine patients. Paschakis and co-workers<sup>15</sup> encountered one case of leucopenia in twenty-one patients. Moore and associates<sup>16</sup> found two cases of leucopenia in fifty-two patients and Bartels<sup>17</sup> only three cases of leucopenia in 119 patients treated preoperatively. Palmer<sup>18</sup> observed nine instances of transitory leucopenia in fifty patients, and Beardwood and Levinson<sup>19</sup> only observed it twice in twenty patients, all of whom required only a temporary discontinuance of the drug. Other authors observed neither agranulocytosis nor leucopenia: Reveno<sup>20</sup> in nine, McGregor<sup>21</sup> in twenty, Watson and Wilcox<sup>22</sup> in eleven, Martin<sup>23</sup> in six, and Nussey<sup>24</sup> in 27.

Nine cases (including the present report) of agranulocytosis have been reported in some detail. Of these, five of the patients have recovered and four have died. These have been summarized in Table I. Six were women and two men, the usual ratio observed in agranulocytosis. The recorded ages were between 37 and 70 years, only one being under the age of 50 years. The length of time thiouracil was administered until agranulocytosis developed was from thirty-five days to slightly more than one year. The daily dose at the time the complication appeared was between 0.1 and 1.0 Gm.; in eight of the

nine patients, it was 0.6 Gm. or less. The total dose of the drug consumed was between 16.8 and 168.3 Gm., but in seven of the nine patients it was less than 50 Gm. In one patient (Case 8) the agranulocytosis appeared during a third course of thiouracil, following rest intervals of four and one-half months and seven weeks. In the other instances the drug was taken without interruption.

TABLE I. SUMMARIZING DATA OF THE PUBLISHED CASES OF AGRANULOCYTOSIS

CASE	REPORTED BY	SEX	AGE (YR.)	DURATION OF TREATMENT	DOSE AT TIME AGRANULOCYTOSIS OCCURRED (GM.)	TOTAL AMOUNT OF THIOURACIL (GM.)	AVERAGE DAILY DOSE (GM.)	OUTCOME
1	Astwood	M	37	35 days	1.0	40	1.14	Recovered
2	McGavack	F	50	42 days	0.5	21	0.5	Recovered
3	Williams	F	?	42 days	0.2	16.8	0.4	Recovered
4	Linsell	F	61	126 days	0.2	49.2	0.39	Recovered
5	Himsworth	F	?	52 days	0.6	64	1.23	Died of broncho-pneumonia
6	Kahn and Stock	F	62	54 days	0.4	30.8	0.57	Died
7	Ferrer and co-workers	M	70	5 mo.	0.6	168.3	1.12	Died
8	Gargill and Lesses	F	56	Little over 1 yr.	0.2	49.4		Died
9	Wosika and Braun	F	50	76 days	0.1	29.4	0.38	Died

Thus, on the basis of this small number of reported cases of agranulocytosis, it would seem that the danger of agranulocytosis is apparently increased in patients over 50 years of age. The duration of the treatment, the size of the daily dose, and the total amount of drug consumed are of no determining importance. However, three received an average daily dose which exceeded 1 Gm. daily for the whole period of treatment, which is definitely higher than the dosages used currently. The time of the onset of the agranulocytosis was between five and eight weeks in five of the nine patients, but the danger of the complication still was present after this time. Usually, the first five weeks are considered the most likely period for the development of complications. It should be noted that in none of the published cases did agranulocytosis develop before the end of the fifth week.

The effectiveness of thiouracil would seem to be established. Close supervision of the patient must be maintained throughout the entire period of its use. Evaluation of the toxicity must await the publication of additional cases with detailed data.

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animal a severe anemia is produced by means of acetylphenylhydrazine or by bleeding, one can observe the launching of many young red cells into the circulation within a few days. If the reticulocytes are counted, the peak of the reticulocyte curve may be used as the starting point for the determination of the lifetime of these new cells.<sup>8</sup> During and after the anemia period the bilirubin excretion is first found to be lower than in the control period; this is due to the change in the age distribution of the red cell population, many young cells being present and, therefore, fewer erythrocytes being destroyed. Then gradually there is an increase of the bile pigment output which reaches its maximum after an average of about 110 to 124 days. This peak may be interpreted as being caused by mass disintegration of the red cells which entered the circulation during the repair period of the anemia and which have now reached the end of their lifetime.

In monkeys, approximately the same average lifetime was found by following the daily reticulocyte count after production of a severe anemia.<sup>9</sup> A second reticulocyte peak representing erythrocytes delivered to replace those which entered the circulation at the time of the anemia was observed after from 94 to 117 days. This second peak was used as the end point in the determinations of the life span.

In man, serologic methods of differential agglutination are now mostly employed for determination of the longevity of the erythrocytes. Ashby<sup>11</sup> was the first to transfuse O erythrocytes into recipients of different blood groups and to determine the time required for the disappearance of these "foreign" cells. This technique has become more refined by using the agglutinogens M and N for tagging of erythrocytes. Wiener<sup>12</sup> and Mollison and Young<sup>13</sup> were thus able to demonstrate that these transfused red cells survive for from 3 to 4 months in the circulation of a normal recipient. With these serologic methods, the maximal survival time of the donor's erythrocytes is estimated. This survival time, however, is not necessarily identical with the true average lifetime of the red cell. In using the method of differential agglutination, at the starting point (day of transfusion) a mixed population of cells of quite different age is introduced into the recipient. The end point (complete disappearance of the test erythrocytes) is not sharply defined, as the assumption that the youngest cells may survive longest is not self-evident. As Baar and Lloyd<sup>14</sup> remark, red cells produced on any day may vary to some extent in their quality and thus cope differently with the fluctuating forces acting in erythrocyte destruction. Thus the average lifetime may sometimes be appreciably shorter than the maximal survival time.

Recently a mathematical analysis of the results obtained in determining the survival time has been performed by British investigators.<sup>10, 15</sup> These workers found that under strictly physiologic conditions there is a gradual disappearance of the transfused red cells and if the numbers of surviving cells are plotted against time in a diagram, a "linear type of decay" is found. This straight line of disappearance depends on a property (age) of the transfused cells themselves. The normal mode of destruction acts so as to remove in the unit of time a fixed fraction of those cells introduced at transfusion. The straight line of decay is, therefore, the expression of the fact that the erythrocytes are destroyed by normal means. As an average, about 0.83 per cent of the transfused cells are removed from the circulation per day; the true average lifetime was, therefore, again computed to be 120 days.<sup>10</sup> If abnormal conditions exist, the line of disappearance of cells becomes curved, thus indicating

that an additional different hemolytic mechanism may be acting concurrently. Such pathologic mechanisms which do not result in a linear line of decay and which change the straight line into a curved one are probably of such a nature that they destroy the erythrocytes at random, irrespective of their age. If, for instance, Rh-positive cells are transfused into a Rh-negative recipient, the physiologic erythrocyte destruction may be interfered with and accelerated by the developing anti-Rh agglutinins. Increased blood destruction may, theoretically, therefore, be caused either by quantitative increase of an already existing physiologic mechanism or by the concurrent presence of qualitatively different processes which condition the pathologic state.

Lloyd<sup>16</sup> and Dacie and Mollison<sup>17</sup> were the first who transfused pathologic red cells from a patient with hemolytic anemia into normal recipients and also exposed normal cells to the apparently abnormal hemolyzing activities existing in the particular patient. One may call this procedure the method of cross-determination of the lifetime of erythrocytes; in this way it may become possible to decide whether the cause of a hemolytic disorder has to be sought for in a structural defect of a particular type of erythrocyte which is responsible for premature disintegration or whether an abnormal hemolyzing activity is present. Thus the various types of hemolytic syndromes may be quite generally classified as being caused either by the presence of an extra- or an intracorpusecular abnormality, or by a combination of both.

#### THE SPLEEN AND HEMOLYSIS

Splenectomy is probably the most successful procedure in the treatment of hemolytic disorders. However, clinical observations demonstrate that excessive hemolysis disappears permanently after splenectomy only in familial spherocytic jaundice and acquired hemolytic anemia<sup>18</sup> but that removal of the spleen is without curative effect in the other hemolytic syndromes. Interpretation of these results of splenectomy is difficult because of scarcity of our knowledge of normal splenic function and of the mechanisms involved in the various types of hemolytic anemias. It is usually assumed that the spleen participates physiologically in erythrocyte disintegration and that in the disorders in which splenectomy is effective, a hyperfunction of the spleen (hemolytic hypersplenism) exists.

Several years ago a study was undertaken of the hematologic data collected from patients who had been splenectomized for various disorders.<sup>1</sup> Patients who had been operated upon for traumatic rupture of the spleen or for thrombocytopenic purpura and who had made a complete recovery were of particular interest, as in these patients no primary disease of the erythropoietic apparatus had been present prior to the operation. In Table I are contained the data of eight of these patients.

In all these patients the quantitative aspects of blood production and delivery were quite satisfactory. There was no anemia and the reticulocyte counts were either normal or slightly elevated. Qualitatively, however, erythrocyte production was not a normal one. All these patients showed Howell-Jolly bodies and target cells (leptocytes), that is, red cells, thinner than normal ones and characterized by a decreased fragility to hypotonic saline solutions. Blood destruction seemed to be diminished. The hemolytic index was definitely, and sometimes considerably, lower than normal in seven of the eight patients. This was first interpreted as a confirmation of the hypothesis that the spleen is engaged in the destruction of normal red cells.<sup>1</sup> According to the int...



TABLE I. HEMATOLOGIC DATA OF CASES SPLENECTOMIZED FOR NONHEMOLYTIC CONDITIONS

CURRENT NUMBER	SEX	AGE (YR.)	DIAGNOSIS	TIME AFTER SPLENECTOMY	HEMOGLOBIN		R.R.C. (MILLIONS PER C.M.M.)	RETICULOCYTES (PER CENT)	HOWELL-JOLLY BODIES (PER CENT)	TARGET CELLS (PER CENT)	HYPOTONIC FRAGILITY % NaCl SOLUTION		SERUM BILIRUBIN (MG./100 C.C.)	FECAL UROBILINOGEN (MG./DAY)	HEMOLYTIC INDEX
					GM.	PER CENT					BEGINS	COMPLETE			
1	M	24	Traumatic rupture	9 mo.	14.9	96	4.9	0.8	0.4	9.6	.40	.08	0.68	55.0	8.0
2	F	35	Purpura	18 mo.	14.3	92	4.9	1.4	0.7	2.7	.42	.12	0.77	37.5	6.5
3	F	50	Purpura	3 yr.	13.4	86	4.6	1.5	0.5	0.5	.42	.12	0.33	61.4	11.4
4	F	18	Purpura	3 yr.	14.2	91	4.6	0.5	2.2	3.4	.44	.12	0.37	45.8	6.1
5	F	38	Purpura	8 mo.	14.7	95	4.9	1.1	0.9	4.6	.48	.08	0.26	27.8	4.6
6	M	40	Traumatic rupture	15 yr.	15.9	102	5.3	0.8	1.3	0.2	.46	.20	0.50	72.9	8.9
7	M	45	Purpura	7 wk.	13.0	84	4.8	1.9	0.8	2.7	.48	.12	0.32	22.2	3.4
8	F	25	Purpura	4 wk.	13.2	85	5.3	2.2	0.4	2.4	.40	.12	0.30	25.2	5.6
Average															6.1

pendence of the three factors active in maintaining the normal red cell level, however, the presence of a quantitatively normal production existing simultaneously with diminished destruction could mean only that the lifetime of the erythrocytes in the postsplenectomy state must be prolonged (Fig. 1).

The question arose whether this interpretation could really be the correct one. Was it likely that removal of a normal organ (for instance, after traumatic rupture of the spleen) should lead to the production of erythrocytes which were apparently better equipped to cope with the forces causing disintegration than were normal red cells? In order to solve this problem it was decided to determine the life span of red cells before and after splenectomy. As clinical material suited for this problem was not readily available, the experiments were performed in dogs with renal biliary fistula, using the method of Hawkins and Whipple<sup>7</sup> previously mentioned. These experiments are published in detail elsewhere.<sup>8</sup> As can be seen from Table II, the average life cycle before and after splenectomy was practically identical.

TABLE II. AVERAGE LIFE CYCLE OF THE ERYTHROCYTE BEFORE AND AFTER SPLENECTOMY

DOG	BEFORE SPLENECTOMY (DAYS)	DOG	AFTER SPLENECTOMY (DAYS)
1	115	4	110
2	108	5	96
3	117	6	109
Average	113	Average	105

However, the red cells in the postsplenectomy state were not normal, as numerous target cells showing increased saline resistance were present. These target cells thus have the same lifetime as normal erythrocytes.

The results of these experiments are of significance to two often discussed problems: namely, the role of the spleen in physiologic erythrocyte disintegration,<sup>19-21</sup> and the explanation of the effect of splenectomy only, in some of the

hemolytic disorders.<sup>22</sup> Demonstration of the almost identical *avéragé* life cycle before and after splenectomy seems to indicate that "active" participation of the spleen in the physiologic breakdown processes of erythrocytes is either negligible or absent or can be entirely compensated by extrasplenic activities. Lauda,<sup>21</sup> in his monograph on the physiology of the spleen, has critically reviewed all the evidence in support of splenic hemolysis. According to this author it is necessary to differentiate between passive and active hemolysis of the spleen. There is no doubt that this organ takes part in removing the debris of red cells which have already disintegrated in the circulation. The spleen functions as the lymph node of the blood. The concept, however, that the spleen actively removes or destroys red cells under physiologic conditions does not withstand rigorous criticism in Lauda's opinion.

Red cells are thought to be destroyed by phagocytosis, fragmentation, and erythrolysis. The latter may possibly be enhanced by the fat content of the food.<sup>23</sup> As the breakdown of red cells continues after splenectomy, it is evident that the presumed participation of the spleen in this mechanism can only be one feature of the general problem of the means and ways of physiologic erythrocyte disintegration. Although erythrophagocytosis has been observed in the spleen, many authorities<sup>21, 24</sup> believe that this mechanism plays hardly any important role in the destruction of red cells, whereas others<sup>25</sup> are still convinced of its significance. Knisely<sup>25</sup> was unable to notice any erythrophagocytosis in the transilluminated living spleen but observed it in the dying animal or after traumatization. Compensatory erythrophagocytosis in other organs belonging to the reticulo-endothelial system is also not demonstrable after splenectomy.<sup>10</sup> Any active influence of the spleen on fragmentation is unknown, and whether tissue or blood lysins (lysolecithin) manufactured in the spleen are of any real significance has not yet been elucidated.<sup>26</sup> There remains only the apparent influence of splenectomy on pigment metabolism as positive proof for a physiologic splenic hemolytic function. The demonstration of a low hemolytic index after splenectomy may, however, require a different explanation.

The values of the hemolytic index in human beings are not based on the determination of bilirubin but on its derivatives. Investigations in the field of pyrrol chemistry during the last years have revealed the great complexity of the various phases of pigment metabolism. In the diagram on the following page are summarized the three main pathways of hemoglobin disintegration.

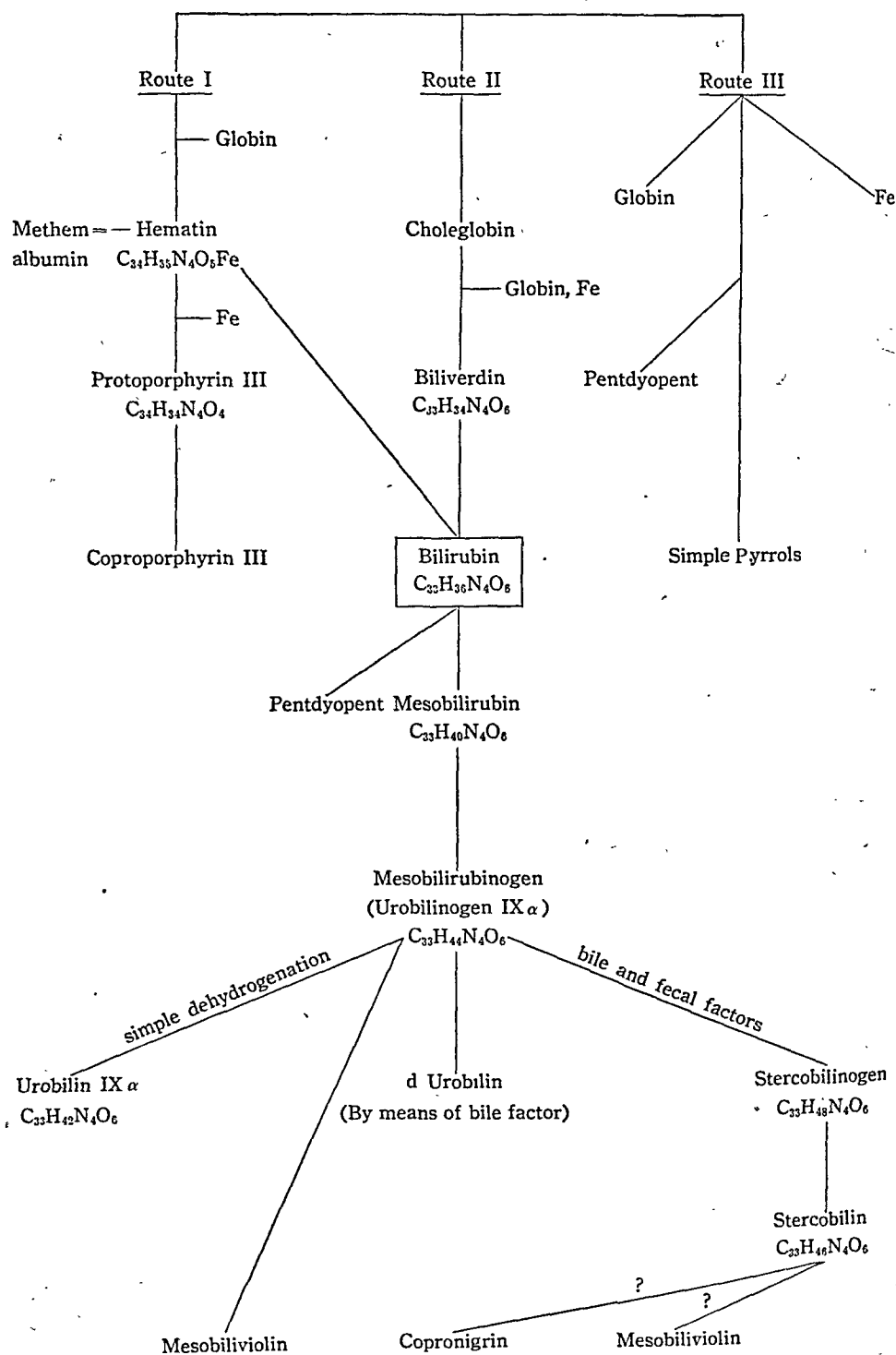
Route I is used when hemoglobin is broken down due to bacterial contamination. First the globin is removed and hematin formed. According to Farley,<sup>27</sup> hematin is immediately combined with the crystal albumin of the plasma to form methemalbumin. Whether hematin can be converted in the organism into bilirubin is still a moot question.<sup>28 \*</sup> After the iron is split off, protoporphyrin results.

The second pathway is probably used in the body for the breakdown of hemoglobin leading to bilirubin. Lemberg<sup>29, 30</sup> has demonstrated that hemoglobin in the presence of ascorbic acid and molecular oxygen is transformed to choleglobin which has an open porphyrin ring. The first globin- and iron-free pigment is biliverdin, which, however, is rapidly reduced to bilirubin.

Whether the third pathway, where the hemoglobin molecule disintegrates into simple pyrrols or dipyrrols besides iron and globin, has any physiologic significance has not yet been demonstrated. This breakdown was accom-

\*Recently Pass, Schwartz, and Watson (*J. Clin. Investigation* 24: 283, 1945) found a proportional augmentation of fecal pigment after intravenous administration of hematin in human beings. This may be interpreted as evidence of formation of bile pigment from hematin. However, hematin does not seem to be a physiologic precursor of bilirubin.

# HEMOGLOBIN (Ferroprotoporphyringlobin)



plished *in vitro* by Haurowitz and co-workers<sup>31</sup> by means of the vitally important linoleic and linolenic acids in the presence of oxygen.

As hemoglobin is, to a very large extent, quantitatively transformed to bilirubin, determination of this latter pigment should give a reliable indication of the degree of hemoglobin destruction. However, such determinations are not feasible in human beings. Quantitative recovery of bile pigment derivatives is usually employed instead in clinical investigations. Watson's<sup>28</sup> excellent studies have recently demonstrated that the bilirubin derivatives to be encountered in feces and urine are, however, much more numerous than previously believed and that at present they can only partly be quantitatively determined. The results of such investigations of bilirubin derivatives are, therefore, definitely inaccurate. Only if all the bile pigment derivatives stemming from bilirubin could be quantitatively recovered could a truly exact picture of the state of hemoglobin destruction in human beings be obtained. It may be suggested that all derivatives of bilirubin found in the excreta be called "exobilins." The exobilins may then be divided further into (1) bile pigment derivatives which, at the present time, can be quantitatively determined, and (2) into substances, like mesobiliviolin,<sup>32</sup> copronigrin, and probably many others, for which no quantitative methods are yet available. It is obvious that this fact must influence the interpretation of the hemolytic index. An index higher than normal is almost always indicative of an increase of blood destruction; an index lower than normal, however, may be due (1) to diminished bilirubin formation, (2) to a sparing action of the body, or (3) to a change in the ratio normally existing between the quantitatively determinable and nondeterminable exobilins in favor of the latter.<sup>2</sup> It is hypothesized that the lower hemolytic index found after splenectomy may be explained in such a fashion, although no actual evidence for this assumption is available at present. In dogs with renal biliary fistula the bilirubin excretion is not significantly altered by splenectomy.<sup>8</sup>

The possibility that the lifetime of the erythrocyte before and after splenectomy may be identical in dogs only, but not in human beings, does not seem very likely, as the hematologic changes occurring after splenectomy are identical in both species. Further combined studies of the survival time of transfused erythrocytes and the pigment metabolism in splenectomized human beings are necessary to elucidate this problem.

Considering the lack of any positive evidence that under physiologic conditions the spleen participates in erythrocyte disintegration and assuming that in man as well as in dogs the average life span of the erythrocyte after splenectomy is normal, the hypothesis may be advanced that physiologically splenic hemolysis is absent or insignificant. There can be, however, no doubt that under pathological conditions splenic hemolysis does exist. It is selective, conditioned by abnormal properties of the red cells which circulate through the organ.<sup>8</sup>

In familial hemolytic jaundice abnormal erythrocytes (spherocytes) are present which continue to exist after splenectomy. The increased saline fragility of these red cells may stay unaltered or become slightly diminished after removal of the spleen but it still remains within pathologic range.<sup>22</sup> In spite of the continuous presence of abnormally shaped cells, excessive hemolysis ceases abruptly after splenectomy and pigment metabolism becomes entirely normal. The most astonishing feature in this return to normal, often not sufficiently appreciated, is the fact that the extrasplenic "hemolyzing activities" of the body treat the spherocytes in a normal manner. Continuation

of abnormal hemolysis after splenectomy is extremely rare and always accompanied by other atypical features. The spherocytes in familial hemolytic jaundice are, therefore, able to fulfill their function as hemoglobin carriers in the absence of the spleen. Increased function of the spleen, hemolytic hypersplenism, was supposed to explain this phenomenon. Recently, however, cross-determinations of the lifetime of the erythrocytes have rendered this explanation untenable. Lloyd<sup>16</sup> and Dacie and Mollison<sup>17</sup> demonstrated that spherocytes transfused into normal recipients were rapidly destroyed, whereas normal cells transfused into patients with familial hemolytic anemia survived normally. These experiments show that the normal spleen destroys the abnormal cells selectively and that the supposedly pathologic spleen does not attack the normal cells in an abnormal way. No hemolytic "hypersplenism" is, therefore, demonstrable in familial hemolytic jaundice. Some abnormality residing inside the red cells seems to be responsible for their disintegration in or through the spleen.

Not only spherocytes, however, but other abnormal cells as well may be eligible for splenic hemolysis.

Before the discovery of liver treatment, splenectomy was recommended in pernicious anemia. It had no curative effect but was often followed by a transient improvement of the anemia. Eppinger<sup>33</sup> investigated the bile pigment metabolism before and after splenectomy in his patients with pernicious anemia and found a marked diminution of the urobilinogen output after removal of the spleen simultaneously occurring with the improvement of the anemia. Whether these findings are really indicative of splenic hemolysis of the megalocytes requires further studies. That the circulating erythrocytes in pernicious anemia have undoubtedly a much shorter lifetime than normal cells was demonstrated by Morawitz.<sup>34</sup> He transfused normal erythrocytes into a nonsplenectomized case of pernicious anemia to such an extent that the majority of the red cells in the patient's circulation belonged to the donor's group. As the anemia improved for a longer period of time, but no change in the pigment output became noticeable, it is evident that the transfused normal erythrocytes survived much longer than the pathologic megalocytes.

In acquired hemolytic anemia the red cells may or may not be spherocytic and may or may not be more fragile to hypotonic saline than normal ones.<sup>35</sup> Splenectomy often cures these syndromes and if spherocytes are present, they may disappear permanently after removal of the spleen in contradistinction to familial hemolytic jaundice.<sup>35</sup> From the standpoint of our hypothesis, these "splenogenic spherocytes" must first undergo their change in shape in order to become liable for splenic destruction.

In sickle-cell anemia, in the Mediterranean syndromes, and in nocturnal hemoglobinuria, the erythrocytes are also definitely pathologic,<sup>36</sup> but the corpuscular abnormality making them eligible for splenic hemolysis appears to be lacking as can be deduced from the absence of any effect of this operation in these conditions. Only by a better understanding of the nature of the red cell abnormalities which induce splenic hemolysis will this problem be further clarified.

#### DEFECTS IN THE STRUCTURE OF THE RED CELL AS CAUSES OF HEMOLYTIC ANEMIA THE DIFFERENTIAL FRAGILITY TEST

We are accustomed to considering the physiologic destruction of erythrocytes as being caused by some "activities" of the body. Be that as it may (and as already pointed out, very little is known about it), the determinations of

the lifetime of the red cells have clearly demonstrated that erythrocyte disintegration also depends on their age. The linear type of decay of transfused cells in man, as noticed by Callender, Powell, and Witts,<sup>10</sup> and the mass disintegration after from 100 to 120 days of red cells launched for the regeneration of an anemia in animals<sup>7-9</sup> are sufficient evidence to support such a statement. Aging of the cell means physicochemical changes in its structure or stroma and apparently if these alterations progress far enough the cells break up. Under pathologic conditions mechanisms may be at work which produce either structural abnormalities in primarily intact cells or red cells which may from the beginning of their life be characterized by certain defects. Such alterations may then lessen their resistance to the vicissitudes of life in the circulation, thus shortening the life span and, therefore, increase the rate of hemolysis. In Table III a classification of the hemolytic syndromes is attempted, based on our present knowledge as to whether extra- or intracorpuseular abnormalities are responsible for the premature disintegration of the cells.

TABLE III. CLASSIFICATION OF HEMOLYTIC SYNDROMES PREDOMINANTLY DUE TO EXTRACORPUSCULAR ABNORMALITIES, INTRACORPUSCULAR ABNORMALITIES, AND UNKNOWN MECHANISMS

- 
- |    |   |
|----|---|
| A. | Extracorpuseular Abnormalities  |
|    | 1. Protozoal and bacterial disease: malaria, bartonella, infections with <i>Bacillus welchii</i> , <i>Streptococcus hemolyticus</i> and <i>viridans</i> , etc.                      |
|    | 2. Chemical agents: phenylhydrazine, arseniureted hydrogen, sulfa drugs, lead, snake venoms, etc.   |
|    | 3. Favism   |
|    | 4. Immune bodies (agglutinins and hemolysins): transfusion accidents, hemolytic anemias, paroxysmal cold hemoglobinuria, hemolytic anemia of the newborn (erythroblastosis fetalis) |
|    | 5. Blackwater fever   |
|    | 6. Physical agents: heat, severe burns  |
| B. | Intracorpuseular Abnormalities  |
|    | 1. Familial spherocytic hemolytic jaundice  |
|    | 2. Sick cell anemia   |
|    | 3. Cooley's anemia and the related Mediterranean syndromes  |
|    | 4. Marchiafava-Micheli syndrome: paroxysmal nocturnal hemoglobinuria  |
|    | 5. Pernicious anemia  |
|    | 6. Certain cases of acquired hemolytic anemia responding to splenectomy?  |
| C. | Unknown Mechanisms  |
|    | 1. Certain acquired hemolytic anemias of unknown etiology not responding to splenectomy   |
|    | 2. Symptomatic hemolytic anemia occurring in neoplasms, leucemias, Hodgkin's disease, etc.  |
- 

The common denominator in almost all the conditions classified in Table III as caused by extracorpuseular anomalies is the fact that a great variety of substances of different types can actively injure the red cells. Such an injury (1) may lead to the immediate dissolution of the erythrocytes in the blood stream (hemoglobinemia) or in the organs belonging to the reticulo-endothelial system or (2) it may manifest itself in changes which predispose the red cells to premature disintegration. Aside from the characteristic alterations of the erythrocytes brought about by the malaria plasmodia, certain poisons, for instance, phenylhydrazine, produce the so-called Heinz bodies, highly refractile crystallike aggregates which frequently protrude from the side of the cell.<sup>4</sup> Immune bodies or poisons may also produce spherocytosis with a concomitant increase in saline fragility. Dameshek and Schwartz<sup>37</sup> demonstrated experimentally that spherocytosis develops after injection of anti-red cell sera into animals. Normally the red cell is a biconcave disk. This shape makes it necessary to consider the cell as being in a state of permanent strain

(Ponder<sup>38</sup>). When this strain is released, the cell assumes spherical form as the result of the action of surface tension. What physicochemical changes inside the cell cause this release is unknown. It is obvious, however, that globular shape may often be a consequence of some preceding injury to the erythrocyte. Although spherocytosis is sometimes reversible,<sup>39</sup> usually it has to be considered as a forerunner of hemolysis,<sup>40</sup> possibly in relation to the degree of damage to the cell.

Often classification of a hemolytic syndrome will depend on the result of the cross-determination of the lifetime of the erythrocytes. In patients with blackwater fever transfused normal red cells are as rapidly destroyed as the patient's own cells, a convincing proof for the existence of an extracorpuscular mechanism responsible for the pathologic hemolysis.<sup>41</sup> The nature of this hemolysin is unknown. In severe burns destruction of a considerable volume of erythrocytes may result directly from the heating of blood at the site of the burn. Damage of the red cells manifests itself in fragmentation, budding, and spherocytosis.<sup>54</sup>

In the disorders of Group B, the cause of the hemolytic disease is intracorpuscular without any evidence for the presence of an abnormal injurious agent causing the anomaly. However, some of the abnormalities residing in the red cells have the peculiarity of rendering a particular type of erythrocytes vulnerable to specific physiologic conditions which have no deleterious effect on normal erythrocytes. Ham and Dingle<sup>36</sup> demonstrated that the red cell in paroxysmal nocturnal hemoglobinuria is attacked by a serum factor indistinguishable from serum complement. The spherocyte in familial hemolytic jaundice is eligible for hemolysis in or through the spleen which organ does not alter the lifetime of normal cells. The sickling phenomenon of the red cells in sickle-cell disease depends on the CO<sub>2</sub> tension in their environment, a mechanism which is probably of great importance for the clinical manifestations of this disorder.<sup>42</sup>

It is of great interest that in this group the hereditary hemolytic syndromes can be found. As Haden<sup>43</sup> has emphasized, sickle-cell anemia and Mediterranean anemia are diseases in which there is evidently present an inherited defect in quality of the stroma, so that the cells disintegrate prematurely. The mechanisms which produce these defects are unknown.

The megalocyte in pernicious anemia is a red cell which is also abnormal in structure; the cause of this anomaly is now recognized: it is the absence of the antipernicious principle during the development of the cells in the bone marrow. The lifetime of the pernicious anemia erythrocyte is considerably shorter than that of normal cells<sup>34</sup>; therefore, this disorder should also be classified as a hemolytic anemia.

Only a few years ago spherocytosis was considered to be pathognomonic of congenital hemolytic jaundice. This view had to be abandoned when it was shown that spherocytes could be produced experimentally.<sup>38</sup> Based on these experiments, the hypothesis was advanced that spherocytosis in the hereditary disease may be due to the action of a "hemolysin" or metabolite on normal erythrocytes.<sup>45</sup> This theory, however, cannot be reconciled with the fact that normal cells, transfused into patients with hereditary spherocytic jaundice, have a normal longevity.

The alteration which is present in the spherocytic cells in familial hemolytic jaundice only interferes with their lifetime in the presence of the spleen. The globular shape of the erythrocytes was considered to be the cause of their destruction in the spleen. Whipple<sup>44</sup> believes that normal discoidal cells have

no difficulty in traversing this organ, whereas spherocytes, being thicker, are unable to pass through the stomas of the splenic sinuses. However, clinical experience shows that nonspherocytic cells may sometimes also be hemolyzed in the spleen. Ham and Castle<sup>45</sup> regard intravascular stasis as the immediate mechanism of splenic hemolysis, resulting in increased blood destruction in hemolytic anemias characterized by increased saline fragility of the red cells; they demonstrated that the saline fragility of the erythrocytes in the spleen increased in their experimental animals when prolonged stasis was induced by nembutal anesthesia. As Dacie<sup>22</sup> points out, this hypothesis is also difficult to reconcile with the observation that the greatly engorged spleen in spherocytic jaundice destroys spherocytes selectively. Furthermore, increased blood destruction should be demonstrable in all disorders with enlargement and engorgement of the spleen, which, however, is not borne out by clinical observations.

Haden<sup>46</sup> has admirably correlated the changes in shape of the erythrocytes with the changes in hypotonic fragility. Normal erythrocytes, when placed in hypotonic salt solutions, become progressively more globular due to the entrance of water into the cell until the cell ruptures. Spherocytes, being globular to begin with, already have one of the shapes through which normal cells must pass and, therefore, they are nearer to the "hemolysis point." While there is usually a striking parallelism between resistance of the cell to osmotic hemolysis and the degree of spherocytosis, there are exceptions to this rule as Haden himself emphasized.<sup>43</sup> Occasionally one may come across spherocytosis with normal fragility<sup>40, 43</sup> which may be due to increased resistance of the cell envelope, or one may see little spheroidicity with greatly increased fragility.<sup>43</sup> The physicochemical factors which influence the strength of the cell membrane or the resistance of the stroma to release the strain inside the cell are unknown. However, such deviations from the rule again demonstrate that increased spheroidicity may often be only one of the manifestations of more general cell defects; the latter probably are of much greater pathogenic significance.

That changes in shape and fragility do not necessarily indicate a change in the lifetime expectancy may also be seen from the presence of target cells (leptocytes) with increased saline resistance and a normal lifetime in the postsplenectomy state.<sup>8</sup> Target cells with increased saline resistance are also found in sickle-cell anemia and Cooley's anemia, disorders in which the red cells disintegrate much faster than normally. Recent studies of the members of families in which Cooley's anemia occurred have demonstrated that clinical pictures exist in which numerous target cells are present but without the signs of an increased rate of hemolysis. Transitions may be found to the fully developed, severely hemolytic Mediterranean anemias.<sup>47, 56-58</sup> In these various Mediterranean syndromes, therefore, the leptocytes show a variable tendency to disintegration, which is not directly related to the shape and fragility of the cells. It is believed that the degree of the existing structural defect of these cells conditions their longevity in the circulation.<sup>58</sup>

Anomalies of red cells may also manifest themselves in a pathologic resistance to agents other than hypotonic, saline solutions. Such studies have been performed with lysolecithin, saponin, and acids, and characteristic reac-

\*This belief is supported by recent (741, 1945). These workers showed that normal erythrocytes in plasma or serum by evaporation. Naturally occurring target cells are not affected.

M. Sc. 209: suspending chemicals or actively normal with disrelation to syndromes cells are con-



tions have been obtained. In Table IV are shown the results of such investigations. These substances may be employed systematically as a diagnostic procedure, thus constituting a "differential fragility test"<sup>48</sup> which is based on the concept that pathologic cells, when injured by different substances, react differently.

TABLE IV. DIFFERENTIAL FRAGILITY TESTS

SUBSTANCE	FINDINGS
Hypotonic saline solutions	Increased in conditions with spherocytosis, such as: Hereditary and acquired hemolytic jaundice Leucoerythroblastic anemia Decreased in conditions with leptocytosis (target cells) such as: Sickle-cell anemia Mediterranean syndromes Postsplenectomy state Severe liver disease Hemorrhage
Lysolecithin (Singer <sup>16</sup> , 40)	Same as with hypotonic saline. Exceptions: blackwater fever <sup>49</sup> —increased lysolecithin fragility but normal saline fragility Acquired hemolytic anemia—normal lysolecithin fragility but increased saline fragility
Saponin (Erlsbacher and Kindermann <sup>50</sup> )	Increased in pernicious anemia
Dilute hydrochloric acid (Ham <sup>51</sup> )	Increased in Marchiafava-Micheli syndrome

Recently Hegglin and Maier<sup>52</sup> demonstrated a diminished "heat resistance" of the erythrocytes in the Marchiafava-Micheli syndrome. When blood is placed in an incubator at 37° C. for six hours, gross hemolysis of the clotted blood is considered specific evidence for the presence of this disorder. According to Minot and Castle,<sup>53</sup> this test does not depend on the heat resistance of the erythrocytes; it is caused by the accelerated acid production at the increased temperature of the incubator. Addition of alkali to the blood inhibits the effect of incubation. Doubtless other substances may be found and used in the future for the performance of the differential fragility test.

Mechanical fragility has also been studied and it was found that pathologic cells break up more easily than do normal ones when exposed to mechanical injury. All these findings again demonstrate that structural alterations of the red cells may also be detected by physicochemical methods.

As can be seen from these discussions, the many factors which may cause pathologic hemolysis seem always to be qualitatively different from those responsible for the physiologic disintegration of the red cells. The useful concept applied in other fields of medicine to explain pathologic phenomena as quantitative deviations from the normal, does not seem to be expedient in the hemolytic syndromes. However, if aging of the cell means physicochemical changes in the structure of the erythrocyte, then physiologic and pathologic disintegration could be correlated in the future when a better knowledge of the various characteristics of cell alterations may be available.

## SUMMARY

There are three interdependent factors active in maintaining the normal red cell count: erythrocyte production, disintegration, and the life span of the red cell in the circulation. Shortening of the lifetime may be due to endogenous factors (i.e., faulty structure of the erythrocyte, abnormal hemolyzing activities), or may be caused by removal of erythrocytes from the circulation (hemorrhage).

With different methods, the true average lifetime was found to be from 100 to 120 days in dogs, monkeys, and man. Under strictly physiologic conditions, the disappearance of the red cells depends on their age. If pathologic mechanisms are at work, they concur with the physiologic processes and destroy the erythrocytes at random, irrespective of their age. The method of cross-determination of the lifetime of the erythrocytes (i.e., the survival time of normal cells in a patient with a hematologic disorder and of this patient's erythrocytes in a normal individual) demonstrates whether the cause of a disorder is due to an extra- or an intracorpuseular abnormality.

The lifetime of the red cells before and after splenectomy in dogs was found to be practically identical. After splenectomy the red cell population contained numerous target cells with increased resistance to hypotonic saline solutions; the longevity of these abnormally shaped cells is not prolonged.

Participation of the spleen in physiologic blood destruction seems to be absent or negligible. In certain hemolytic anemias (familial spherocytic jaundice, acquired hemolytic anemia) splenic hemolysis is present; it is selective, conditioned by abnormal properties of the erythrocytes which circulate through the organ. No evidence is available for the existence of a primary hemolytic "hypersplenism." The diminution of the pigment excretion observed after splenectomy in human beings is not indicative of a decrease of blood destruction.

The hemolytic syndromes may be classified in two main groups: (1) disorders due to a variety of substances which, by acting on the red cells, injure their structure, and (2) diseases in which there are various and characteristic structural defects present in the red cells at the start of their life. The physicochemical changes responsible for the different structural alterations are unknown. Defective cells usually, but not always, have a shortened life span. Some defects make cells vulnerable to physiologic mechanisms.

Structural defects manifest themselves morphologically by changes in size, shape, and volume of the red cells, or functionally in such a way that pathologic cells, when injured by different substances, react differently (differential fragility). The many factors which cause pathologic hemolysis seem always to be qualitatively different from those responsible for the physiologic disintegration of the erythrocytes. However, if aging of the cell means physicochemical change in its structure, then physiologic and pathologic disintegration could be correlated in the future when a better knowledge of the characteristics of the various cell alterations may become available.

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A penicillin-impregnated disk 15 mm. in diameter is then placed on the surface of the culture medium over the central circle. In the Chemosurgery Clinic penicillin ointment-impregnated gauze is kept on hand for the local treatment of infections as described elsewhere,<sup>4</sup> and it is convenient to use this material for the disk. This penicillin gauze is impregnated with a standard amount of an ointment containing 500 Oxford units per gram so the penicillin content of the disk is essentially constant. If the penicillin gauze is not available it is just as satisfactory to place a 15 mm. disk of filter paper on the culture plate and to drop upon it from a No. 20 hypodermic needle one drop of a solution containing 5,000 units per cubic centimeter.

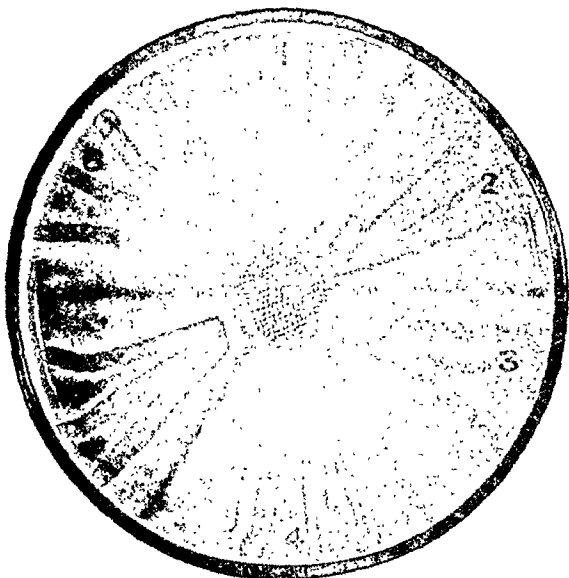


Fig. 1.—Radial streak cu (sectors 1 to 5) in relation to t strain 209-P (sector 5). Taking cent. the sensitivities of the test organism (*Staphylococcus aureus*), 21 mm. or 1

of five test organisms *Staphylococcus aureus*, the control as 100 per cent; sector 1 (*Streptococcus aureus*), 0 mm. or 0 per cent; *Staphylococcus aureus* from a patient on penicillin therapy, 100 per cent; sector 5

The culture plate is then placed in an incubator at 37° C. Usually the cultures are incubated overnight and the zone of inhibition is measured the next day. However, if a reading is wanted sooner, there is usually enough growth by eight hours for a fairly accurate determination of sensitivity.

The distance between the edge of the central circle and the uninhibited portion of the streak is measured with calipers. Taking the inhibition distance exhibited by the control organisms as 100 per cent, the relative sensitivities of the test organisms are expressed in per cent. Thus, if the control organism is inhibited for 13 mm. and the test organism is also inhibited for 13 mm., the sensitivity of the latter is 100 per cent. However, if the test organism is in-

## LABORATORY METHODS

### A SIMPLE QUANTITATIVE TEST FOR THE PENICILLIN SENSITIVITY OF BACTERIA: THE "RADIAL STREAK" METHOD

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WHEN penicillin therapy of an infected lesion is contemplated, it is important to determine, as soon as possible, whether or not the causative organisms are sensitive to the drug. If they are sensitive it is also advantageous to know the degree of sensitivity. A number of sensitivity tests have been devised, some of which are fairly simple.<sup>1, 2</sup> An even more simple quantitative test and one having several other advantages is provided by the "radial streak" method described herewith. This test is based on the original observations of Fleming<sup>3</sup> which indicated that penicillin diffuses through agar, preventing the growth of sensitive organisms for a distance proportional to the susceptibility of the organisms to the drug. In fact, the radial streak test is simply a modification of methods devised by Fleming.

#### MATERIALS AND METHODS

The only material required for the test is a 5 per cent blood agar plate, a disk of penicillin-impregnated gauze or filter paper, and a culture of *Staphylococcus aureus* of known sensitivity to penicillin. On the bottom of the Petri plate is drawn a central circle 15 mm. in diameter with radial lines extending to the edge of the plate, dividing it into six sectors. A stencil to guide the wax pencil used in marking is convenient.

Material directly from lesions or from cultures of the causative organisms are streaked on the blood agar by means of small cotton-tipped applicators. Two or three streaks extending from the edge of the central circle to the edge of the plate are made, keeping well within one sector (Fig. 1). An attempt should be made to use approximately the same amount of infected material for all of the streaks because the number of organisms has a slight effect on the degree of inhibition. It is sometimes impossible to judge accurately the number of organisms in the test material, especially when pus directly from a lesion is being tested. Under these circumstances, varying quantities of the material are used in the two or three streaks made in one sector. If, despite these precautions, the seeding is either too heavy or too light, it is desirable to repeat the test so that a more accurate reading may be obtained. When twenty-four hour cultures of the test organisms are used, this source of inaccuracy is minimal.

In a busy clinic a number of sensitivity tests are usually desired each day so the plate is returned to the refrigerator after each streaking until five sectors have been inoculated. For a control the sixth sector is streaked with a twenty-four-hour broth culture of *Staph. aureus* culture of known sensitivity to penicillin.\*

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\*For uniformity, *Staphylococcus aureus*, strain 209-P, has been adopted as the control culture. This was obtained from the Food and Drug Administration through the kindness of Dr. Albert C. Hunter.

## DISTILLING APPARATUS FOR CLINICAL LABORATORIES

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**D**ISTILLING apparatus of the all-glass type is essential in many procedures and more convenient than ordinary distilling apparatus in other procedures. Among instances that may be cited are preparation of constant boiling hydrochloric acid for standards, purification of hydrochloric and nitric acids for determination of lead and other metals, preparation of conductivity water or water free of metals, purification of alcohol and other organic solvents for use in lipid determinations, recovery of organic solvents, and determinations of alcohol or iodine in blood. The outfits described in this paper have been so designed as to minimize the amount of desk space required, to eliminate waste of time spent in assembling, and to reduce the cost of replacing broken parts. In constructing them, standard Pyrex parts listed in the catalogs of the firms supplying laboratory glassware have been used. The catalog names of parts are used in the following description.

### ALL-GLASS APPARATUS

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All sizes of this outfit up to 3 liters are satisfactorily supported by a single large castaloy extension clamp. The flask may be filled through the funnel, or water to be distilled may be siphoned in through the side arm of the stopcock. This side arm also serves for applying suction to empty the flask or to clean the condenser and flask by reverse washing. Insoluble residues can be removed by admitting hot chromic-sulfuric acid mixture through the funnel. The mixture is subsequently diluted and removed by suction. The outfit can be filled, emptied, and cleaned without removing it from the stand. Besides the purposes already mentioned, the tube within the flask serves to prevent bumping.

From the Department of Laboratories, Henry Ford Hospital.  
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hibited for 6 mm., its sensitivity is 46 per cent ( $6 \div 13 \times 100$ ), while if it is inhibited for 21 mm., its sensitivity is 154 per cent ( $21 \div 13 \times 100$ ).

#### DISCUSSION

Besides giving a quantitative determination of penicillin sensitivity, the radial streak culture also facilitates the identification of the causative organisms. Some bacteria produce characteristic colonies as, for example, *Staph. aureus*, yellow colony with or without hemolysis; *Bacillus pyocyaneus*, blue-green colony; *Streptococcus hemolyticus*, small grayish colony with variable degrees of hemolysis; and *Streptococcus viridans*, green color around colonies. In mixed infections various types of colonies may be selected for further studies by smears and cultures. Differing penicillin sensitivities as shown by Fleming<sup>3</sup> often aid in the separation and identification of the pathogens; for example, a penicillin-resistant organism which would ordinarily be overgrown by a predominating penicillin-sensitive organism will be found in the zone near the penicillin.

Besides being useful in connection with the various surgical specialties, the radial streak test may well have uses in dermatology, medical and pediatric clinics, and even in general practice. The plates may be carried in the physician's bag and inoculated in the patient's home; if smears and further cultures are desired they may be taken from the plate after incubation.

Other advantages of the radial streak test may be mentioned. The economy of time and material occasioned by the use of one plate for five lesions is worth while. The simultaneous inoculation of a control culture of known sensitivity gives reliable determinations without dependence upon standard penicillin solutions which may deteriorate. The exact amount of penicillin in the central disk is less important than in most sensitivity tests because the sensitivities of the unknown organisms are expressed in relation to the sensitivity of the control culture. The facility with which inoculation can be made of material directly from infected lesions permits the determination of sensitivity with minimal delay.

#### CONCLUSION

Testing for the penicillin sensitivity of bacteria is facilitated by the "radial streak" method. The only material needed is a blood agar plate, a penicillin-impregnated disk of gauze or filter paper, and a culture of *Staph. aureus* of known sensitivity.

The author wishes to express his appreciation to Prof. Paul F. Clark, of the Department of Bacteriology, for helpful advice.

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## DISTILLING APPARATUS FOR CLINICAL LABORATORIES

O. H. GAEBLER, PH.D., M.D.

DETROIT, MICH.

**D**ISTILLING apparatus of the all-glass type is essential in many procedures and more convenient than ordinary distilling apparatus in other procedures. Among instances that may be cited are preparation of constant boiling hydrochloric acid for standards, purification of hydrochloric and nitric acids for determination of lead and other metals, preparation of conductivity water or water free of metals, purification of alcohol and other organic solvents for use in lipid determinations, recovery of organic solvents, and determinations of alcohol or iodine in blood. The outfits described in this paper have been so designed as to minimize the amount of desk space required, to eliminate waste of time spent in assembling, and to reduce the cost of replacing broken parts. In constructing them, standard Pyrex parts listed in the catalogs of the firms supplying laboratory glassware have been used. The catalog names of parts are used in the following description.

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All sizes of this outfit up to 3 liters are satisfactorily supported by a single large castaloy extension clamp. The flask may be filled through the funnel, or water to be distilled may be siphoned in through the side arm of the stopcock. This side arm also serves for applying suction to empty the flask or to clean the condenser and flask by reverse washing. Insoluble residues can be removed by admitting hot chromic-sulfuric acid mixture through the funnel. The mixture is subsequently diluted and removed by suction. The outfit can be filled, emptied, and cleaned without removing it from the stand. Besides the <sup>1</sup> poses already mentioned, the tube within the flask serves to prevent bump.

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since it becomes a boiling tube when the upper end is closed by the proper turn of the stopcock. The principle of preventing spray from passing over by means of two inclined tubes and an intervening trap is the same as in the apparatus designed by Yoe.

The 3 liter size has been routinely used for over five years in preparation of conductivity water and constant boiling hydrochloric acid, or for purification of acids. By redistilling ordinary distilled water alone or after addition of alkali and permanganate, water with conductivity of  $1 \times 10^{-6}$  is regularly obtained if the first fourth of the distillate is discarded. The 500 c.c. size has been used in determination of alcohol or iodine in blood. In the latter determination a trace of chromium is carried over, since the concentration of chromic salt in the

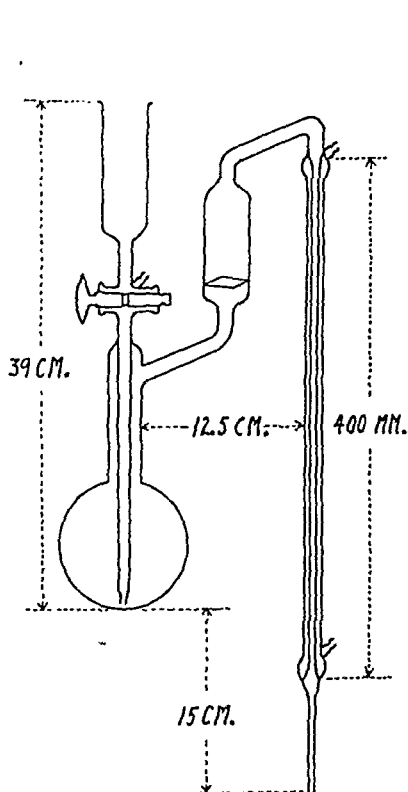


Fig. 1.

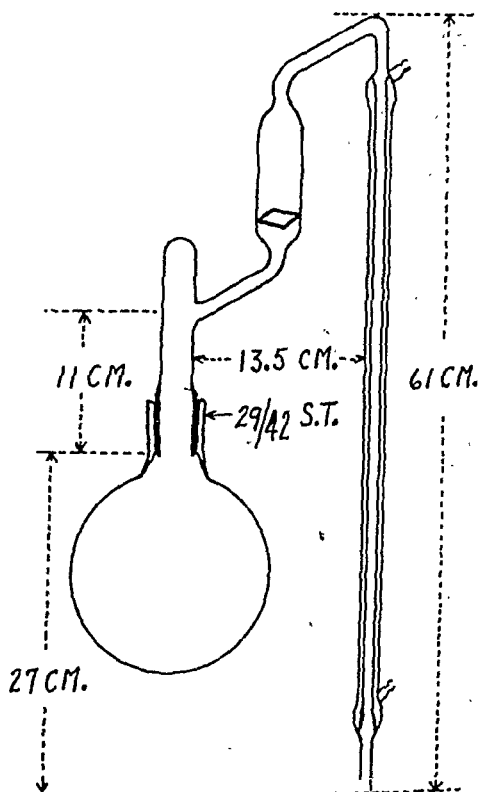


Fig. 2.

flask is very great. Interference of chromate in the final titration of iodate can, however, be prevented.\* Vertical outfits like those shown in Figs. 1 and 2 occupy only a twelve- to fifteen-inch square of desk space. The disadvantage that the outlet of the condenser is near the flame is easily overcome by using a burner shield, or, in the case of inflammable liquids, an adapter which will convey the condensed liquid to a receiver on the floor. The adapter is connected to the condenser with a one-hole stopper with which the distillate does not come in contact.

In designing the two-piece outfit shown in Fig. 2, advantage was taken of the fact that Pyrex round-bottom and flat-bottom flasks of all capacities from 1 to 3 liters are regularly supplied with the same size of standard taper joint.

\*Gaebler, O. H., and Baty, M.: Detection and Titration of Chromate in Blood Iodine Determinations, *Indust. Engin. & Chem. (Anal. Ed.)* 15: 442, 1942.

Of the 1 liter size, the type with a long neck should be used. The other part shown in Fig. 2 is made from the inner cone of a 29/42 standard taper joint, a Kjeldahl connecting bulb, and a West improved type condenser. The inlet and outlet of the condenser extend back at right angles from the plane of the apparatus. As shown in Fig. 2, the inner cone of the standard taper joint has been sealed off at the upper end. A dropping funnel or other equipment can readily be sealed in at this point if desired.

Reference to catalogs will show that the standard flasks used in the present outfit cost only a fourth as much as special flasks. The cost of all parts indicates that the apparatus shown in Fig. 2, with three flasks of different sizes, should be less expensive than another outfit which has only one flask and no trap.

#### MICRO-KJELDAHL DISTILLING APPARATUS

For class use, an inexpensive type of apparatus which will not suck back when the flame blows about is required. The design shown in Fig. 3 meets these requirements. When the amount of nitrogen is between 1.0 and 5.0 mg., results are so precise that the expensive forms of apparatus advertised for this simple procedure seem unjustified.

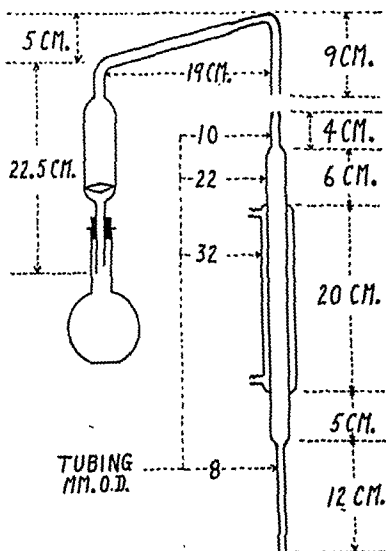


Fig. 3.

The flask is a 250 c.c. Pyrex boiling flask. A No. 4 pure gum amber, or other alkali-resisting type of stopper, must be used. The Kjeldahl connecting bulb has been altered by bending the upper tube to the indicated angle and sealing on a piece of 10 mm. tubing. The latter is bent downward to connect with a condenser through a piece of rubber tubing not shown in Fig. 3. The capacity of the inner tube of the condenser exceeds the usual volume of acid or distillate present above the delivery tip; hence the solution in the receiver can not suck

back. The 4 cm. tip attached to the upper end of the inner tube of the condenser is thickened at its free end to increase its strength and is constricted slightly to facilitate slipping the rubber tubing on and off.

The stopper and that part of the neck of the flask which it enters should be free of alkali and water when the connection is made. The flask is best heated directly, for if a screen is used the heat passing around it converges upon the stopper and soon ruins it. Distillation is stopped by grasping the cold water jacket of the condenser with one hand and working off the rubber connection from the upper end of the condenser with the other.

#### SUMMARY

Distilling apparatus is described which is suitable for the following purposes: preparation of conductivity water, of constant boiling hydrochloric acid, and of water and acids free of lead and other metals, purification or recovery of organic solvents, determination of blood alcohol or blood iodine, and micro-Kjeldahl distillations.

## BOOK REVIEWS

**The Bacterial Cell in Its Relation to Problems of Virulence, Immunity and Chemotherapy.** Harvard University Monograph in Medicine and Public Health No. 6. By *Rene J. Dubos*, recent Professor of Comparative Pathology and Tropical Medicine, Harvard Schools of Medicine and Public Health, and present member of the Rockefeller Institute. Harvard University Press, Cambridge, Mass. Price \$5.00. Cloth with 460 pages.

This excellent volume may well prove to be an important catalyst for stimulating the further development of bacteriology as a biologic science in its own right, as distinguished from its obvious and important utilitarian aims in the fields of medicine, industry, and agriculture.

Due to their microscopic size and absence of recognized sexual mechanism of reproduction, there has arisen an illusion that bacteria possess very primitive simple organization. Actually bacteria perform an amazing multiplicity of functions, and it appears very likely that the failure to recognize in them the corresponding multiplicity of cellular structures underlying these functions is an indication of the deficiency of our techniques rather than an evidence of the simplicity of their organization.

The basic aim of the author is to bring together and to correlate information, which has accumulated as result of various investigations in the fields of bacteriology and immunology, in so far as it tends toward eliciting the "biochemical architecture" of the bacterial cell, and to integrate this information with that secured from the conventional study of bacterial structures by means of cytological techniques.

Inasmuch as this book represents an outgrowth of a course of eight lectures delivered by the author in February, 1944, under the auspices of the Lowell Institute in Boston, the basic contents are presented in eight chapters.

In the first chapter, entitled *Materials, Problems and Methods*, the author emphasizes the questionable validity of any attempt at reconstruction of the trends and direction of evolution in any group of living organisms on morphological grounds alone. It is particularly true of such organisms as bacteria which not only possess very few morphological characteristics useful in classification, but in addition exhibit a high degree of mutability.

In the second chapter, entitled *Cytology of Bacteria*, are discussed data concerning the cell structures as elicited by microscopic examination with visible and ultraviolet light, as well as newer data secured by electronic microscopy.

The third chapter describes *Physicochemical and Staining Properties of Bacteria* with the purpose of eliciting information as to composition and distribution within the cell of various reacting constituents.

The fourth chapter consists of *Analysis of Cellular Structure by Biochemical and Biological Methods* which contributes information as to structure of the bacterial cell. In this connection the author stresses the role of bacterial enzymes and of serological methods as tools for microanalysis. The strict specificity and great reactivity of the later reagents, especially, permits not only the detection of small amounts of specific cellular constituents in the presence of relatively large quantities of contaminating substances, but, in many instances, they also give information concerning the relative position of these components.

In the fifth chapter, *Variability of Bacteria*, the author emphasizes the extraordinary ease with which bacteria adapt themselves to the environment either by reversible modification or by means of *hereditary variation*. This plasticity makes them unique as material for the study of certain problems of genetics, which cannot be investigated with the use of other physiologically more rigid material.

In the sixth chapter, devoted to the discussion of the *Nature of Virulence*, the author emphasizes that virulence is the result of a summation of a number of independent attributes which determine the ability of the parasite to establish itself in the host. Loss of any of these component factors of virulence, as result of variation, tends to diminish or ~~abolish~~ the ability of the parasite to establish a pathological state.

No. 10

In Chapter VII, entitled Immunization Against Bacterial Infection, the author attempts to analyze the present relatively limited information concerning the "protective antigens," i.e., those individual components of the bacterial cell which induce the production of antibodies upon which depends resistance to infection. He brings together the evidence which suggests that the location and orientation of these different antigenic components in the bacterial cell are important in determining the protective value of the antibodies to which they give rise.

In Chapter VIII the author discusses Bacteriostatic and Bactericidal Agents. In this discussion he considers not only the role of specific inhibitors of essential metabolic reactions, but also the enzymic and chemical reagents which can selectively alter or destroy vital morphological structures, as well as the nonspecific physicochemical reactions which affect the protoplasmic constituents of all living cells.

In the ninth chapter, Trends and Perspectives, the author attempts to reconstruct the trend of "bacteriological philosophy" during the present century. Much of the theoretical knowledge of the field of bacteriology and immunology was gathered as a by-product of the efforts to solve practical problems by empirical methods. Frequently, successful practices were developed before there was any understanding of their rational basis. Indeed, it was the analysis of the mode of action of these empirical methods which led to the recognition of a number of reactions between bacteria and the environment and to the subsequent description of the components of the cell involved in these reactions. The author suggests that in order to bring bacteriology to the state of development achieved by other sciences, it is necessary to abandon this anthropocentric attitude which characterized its earlier development—bacteria must be studied not only in the effects which they have on practical human problems, but also for what they are and what they do as independent living organisms.

Since the purpose of the book is to bring together the knowledge of cellular organization, the author appropriately invited Dr. C. F. Robinson to contribute a chapter on Nuclear Apparatus and Cell Structure of Rod-Shaped Bacteria, which reviews the factors which have delayed recognition of morphological organization of bacterial cells and describes some of the newer findings made possible through the employment of newer cytological procedures.

Although the text is replete with technical information, which will be of especial interest to bacteriologists and immunologists, the author has supplied the necessary background so skillfully that even the most technical portions of the text will present no difficulty for biologists outside of the special field of bacteriology.

J. BRONFENBRENNER

## PENICILLIN LEVELS IN SERUM AND IN SOME BODY FLUIDS DURING SYSTEMIC AND LOCAL THERAPY

EDWIN M. ORY, M.D., MANSON MEADS, M.D., BRUCE BROWN, M.D.,  
CLARE WILCOX, AND MAXWELL FINLAND, M.D.  
BOSTON, MASS.

THE rational choice of dosage and route of administration of penicillin presupposes some acquaintance with the effective concentrations of the antibiotic which can be attained and maintained at the site of the infection by the different methods. The desired concentration in any given case will depend, of course, on the susceptibility of the infecting organism. The variations in the sensitivity observed among different strains of some of the commonly encountered bacteria were considered elsewhere.<sup>1</sup> The present paper deals with the concentrations of penicillin obtained in serum and in certain body fluids of adult medical patients after administration of various doses of commercial penicillin given by different routes.

### MATERIALS AND METHODS

Almost all of the observations included in this report were made on materials obtained during the course of treatment of infections on the medical wards. The penicillin was usually given in 0.85 per cent sodium chloride solution except in some of the cardiac patients, in whom continuous injections were given in 5 per cent dextrose solutions, and in those treated by inhalation. The concentration of penicillin in body fluids was determined by the serial dilution method of Rammelkamp.<sup>2</sup> A strain of hemolytic streptococcus No. 98, obtained from Dr. C. S. Keefer, was used throughout. Broth cultures seeded with approximately 10,000 of these streptococci per cubic centimeter were quite consistently sterilized by 0.0078 unit of commercial penicillin. In control tests in which the penicillin solution was contained in a volume of 0.2 c.c. and added to 0.5 c.c. of culture, the mixture was regularly sterilized by 0.0056 unit per cubic centimeter. Human Group O cells were used as an indicator in tests with body fluids in order to avoid the nonspecific hemolysis frequently observed when horse cells were used. Penicillin solutions for the standard controls were prepared at intervals from pools of five vials of commercial penicillin in sterile saline and stored at 5° C. The smallest amount of body fluid tested was usually 0.2 c.c. in a final volume of 0.7 c.c. of the culture. The smallest concentration measured in this manner was approximately 0.03 unit per cubic centimeter. End points were based on the smallest concentration of fluid in which there was no evidence of growth at eighteen hours and which yielded no growth in transplants on blood agar after another twenty-four hours. The method is subject to the wide limits of error common to such biologic tests.

### SERUM LEVELS DURING CONTINUOUS INFUSIONS

Administration of penicillin by continuous infusions has the advantage of permitting the maintenance of fairly constant blood levels as well as avoiding repeated punctures. It does, however, require constant supervision. The intravenous route also necessitates the injection of larger volumes of fluid than

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School.

Part of the penicillin used in this study was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

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are desirable in some of the types of cases in which it is best indicated and, in addition, has given rise to thrombophlebitis in a large percentage of the patients in whom injections were continued for more than forty-eight hours. The continuous intramuscular infusions are somewhat more difficult to regulate and are often quite painful, but they have proved somewhat more satisfactory in this clinic. Both methods are difficult to carry out properly in patients who are irrational and acutely ill. Neither method has been used routinely except when it was necessary to maintain high levels over a long period and when frequent injections were not well tolerated.

Serum levels under continuous infusion by either route did not usually exceed the average concentrations obtained during intermittent intramuscular injections of the same total daily dose, but given every two hours provided that a large fluid intake was avoided. Two examples may be cited from patients with bacterial endocarditis. In one of them, while receiving two-hour intramuscular injections of 15,000 units, the serum levels were 0.22, 0.22, 0.11, and 0.06 unit per cubic centimeter at intervals of one-fourth, one-half, one, and two hours, respectively, following the injections. During a long period of continuous intravenous infusion of the same total daily dose, the levels were usually 0.11 and only occasionally 0.22 unit per cubic centimeter. The second patient had serum levels of 0.22, 0.11, 0.11, and 0.06 unit per cubic centimeter at intervals of one-half, one, one and one-half, and two hours, respectively, after two-hour injections of 25,000 units, but the serum levels during continuous intravenous infusion of 300,000 units daily were repeatedly found to be only 0.06 unit per cubic centimeter. Thus, considerable variations were noted in different patients receiving intravenous infusions. In one instance, detectable levels could not be obtained at all during constant intravenous infusion of 100,000 units a day, while another maintained a serum level of 0.11 unit while receiving 6,000 units per hour intravenously.

Continuous intramuscular infusions also resulted in levels which were quite constant in any given patient but varied in different patients. For example, in one patient receiving 200,000 units daily for a cellulitis, successive serum levels were always 0.06 unit per cubic centimeter. A second patient who received 250,000 units a day for what proved to be miliary tuberculosis regularly had levels of 0.45 unit per cubic centimeter. A patient treated for subacute bacterial endocarditis with 300,000 units a day and tested on sixteen different days was found to have a level of 0.11 on eleven occasions, 0.22 on three, and 0.06 twice. In another similar case on the same dosage, a level of 0.11 was obtained repeatedly.

*Continuous Infusion in a Case of Anuria.*—A patient who developed anuria during the course of sulfonamide therapy for pneumonia offered an opportunity for a study of the cumulative effect of continuous penicillin injection. He was given 100,000 units a day by a constant intravenous drip. The serum levels on four successive days after this was started were 0.22, 0.45, 0.90, and 1.8 units per cubic centimeter. Urine flow was re-established and penicillin was stopped soon after the last sample was obtained. There was no detectable penicillin (that is, less than 0.03 unit per cubic centimeter) in the serum obtained eighteen hours later.

#### SERUM LEVELS DURING INTERMITTENT INTRAMUSCULAR THERAPY

Penicillin concentrations in sera obtained at different intervals after intramuscular injections of 15,000, 20,000, and 25,000 units given at two-hour inter-

vals and after 15,000 or 20,000 units given at three-hour intervals are shown in Fig. 1. Most of these patients had good renal function and were not restricted with respect to their fluid intake but they took only moderate amounts. A few observations, specially designated in Fig. 1, were made on patients who had congestive heart failure. The latter patients were on a restricted fluid regime and had low urinary outputs.

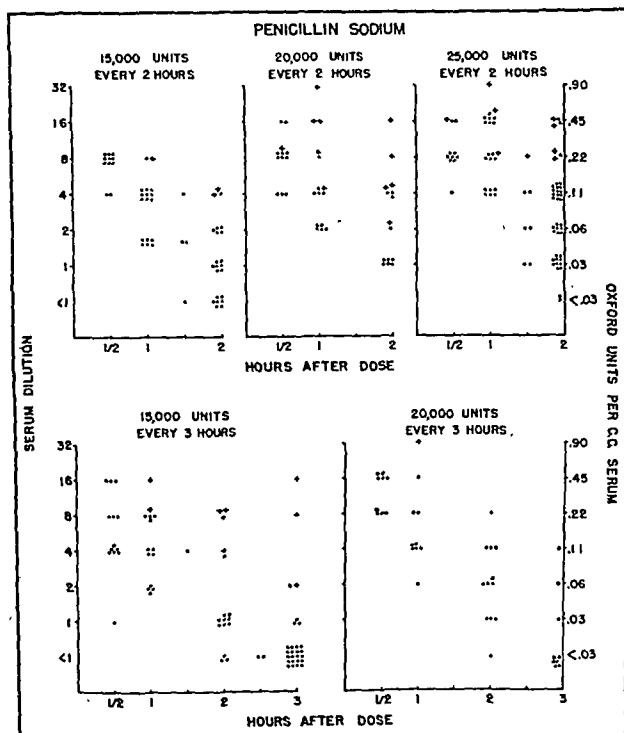


Fig. 1.—Penicillin levels in the serum during intermittent intramuscular administration of commercial penicillin. The dots represent single observations in patients other than those with congestive cardiac failure. Observations in the latter are indicated by +.

In the patients without congestive cardiac failure it is seen that the levels obtained at any given interval after each dose fall within a fairly narrow range. The least variations were observed in the sera obtained at the one-half hour interval in patients who were being treated every two hours. The levels obtained at that time, moreover, were not much different with each of the dosage schedules that were studied in these cases. When 15,000 or 20,000 units were given every three hours, almost all of the sera obtained just before an injection failed to show detectable amounts of penicillin, and only an occasional one showed the smallest measurable amount, namely, 0.03 unit per cubic centimeter. Higher levels at one-half hour were more frequent after the 20,000 unit doses.

The greatest variations were noted in the sera obtained two hours after the injections. On the two-hour schedule about one-third of the sera obtained just before an injection of 15,000 units failed to show detectable amounts of penicillin; a similar number showed the smallest concentration measurable and the rest had twice that amount. With larger doses given every two hours, penicillin was found in almost every serum obtained before an injection. A greater proportion of the levels obtained at that time were higher on the 25,000 unit doses than on the lower amounts. The levels obtained one hour after the various doses also showed a general upward trend with increasing dosage.

Briefly, therefore, in febrile patients on a moderate but unrestricted fluid intake and with adequate renal function, there is less than 0.03 of a unit of penicillin per cubic centimeter of serum three hours after an intramuscular injection of 15,000 or 20,000 units. The same is true in one-third of the sera obtained two hours after the smaller dose. Larger doses give higher and more sustained levels.

In the patients with congestive cardiac failure the levels were uniformly more sustained and at significantly higher levels. The penicillin concentrations obtained in the serum three hours after a 15,000 unit injection in such patients were about comparable with the values obtained two hours after a 25,000 unit injection in ordinary cases.

#### PENICILLIN LEVELS IN CEREBROSPINAL FLUID

Some workers have failed to detect significant amounts of penicillin in cerebrospinal fluid after intravenous or intramuscular injections,<sup>3-6</sup> while others found appreciable concentrations, especially after very large doses in cases of meningitis.<sup>7-10</sup> In the absence of a block, diffusion of the penicillin usually takes place from the lumbar to the cerebral subarachnoid space and into the ventricles<sup>3, 10</sup> as well as in the opposite direction.<sup>9</sup> Penicillin has been detected in the spinal fluid for twenty-four hours and sometimes as long as ninety-six hours after injection by the lumbar route,<sup>6, 10</sup> but occasionally it cannot be detected even after fourteen hours.<sup>9</sup>

In the present studies cerebrospinal fluid was obtained by lumbar puncture in eighteen patients who had received one or more injections of penicillin intramuscularly. All but four of these patients had purulent meningitis. The lumbar puncture was done from one to two hours after an intramuscular dose of from 20,000 to 60,000 units, and venous blood was obtained at the same time. The levels of penicillin in the serum varied from 0.06 to 0.9 unit per cubic centimeter, more than half of them being 0.22 or 0.45 unit. Not one of the spinal fluids taken at the same time showed detectable levels of penicillin.

In thirteen patients under treatment for meningitis, concentrations of penicillin were also determined in spinal fluid obtained twelve or twenty-four hours after intraspinal injections of 10,000 or 15,000 units. The results are shown on Table I. The penicillin was given in a concentration of 1,000 units per cubic centimeter of saline. At twelve hours the levels in nine instances ranged from 10 to 40 units per cubic centimeter and, in one instance, 160 units per cubic centimeter were found. The levels at twenty-four hours ranged between 0.03 and 5.0 unit per cubic centimeter in eighteen instances, more than half of these being less than 1.0 unit. In one instance 80 units per cubic centimeter were found in the fluid at this time. At forty-eight hours, penicillin could not be detected in one case while intramuscular therapy was being continued.

In one patient who died twenty-four hours after a lumbar injection of 10,000 units, the fluid in the lumbar region contained 0.11 unit per cubic centi-

TABLE I. PENICILLIN LEVELS IN CEREBROSPINAL FLUID AFTER INTRASPINAL INJECTIONS

CASE	DOSE (UNITS)	INTERVAL (HOURS)	C. S. F. LEVEL (UNITS PER C.C.)
1	10,000	12	10.00
	10,000	12	40.00*
2	10,000	12	10.00
3	10,000	24	5.00
4	10,000	24	0.03
	10,000	24	0.11
5	15,000	12	40.00
	15,000	12	20.00
	15,000	12	160.00
	15,000	12	10.00
	15,000	24	80.00
	15,000	12	10.00
	15,000	24	5.00
	15,000	24	0.90
	15,000	24	0.06
	10,000	48	0.00
6	15,000	24	1.80
	15,000	12	10.00
	15,000	24	1.8
	15,000	24	0.22
	15,000	24	0.06
	15,000	24	0.90
7	15,000	24	0.22
		24	0.11†
8	15,000	12	20.00
9	15,000	24	2.5
10	15,000	24	0.06
11	15,000	24	2.5
12	15,000	24	1.8
13	15,000	24	0.22

\*Ventricular fluid at this time had 0.9 unit per cubic centimeter.

†Obtained after death. No penicillin was detectable in the cisternal or ventricular fluid at this time.

meter and none was detected in the cisternal or ventricular fluid at autopsy. In a second patient who had a brain tumor, the spinal fluid twelve hours after an intraspinal injection of 10,000 units had 40 units per cubic centimeter and the ventricular fluid had 0.9 unit per cubic centimeter.

The reactions following intraspinal injections were similar to those noted by Rammelkamp and Keefer.<sup>11</sup> In three patients without meningitis receiving from 5,000 to 15,000 units one or two times, no symptoms resulted, and the only abnormality in the spinal fluid noted twenty-four hours later consisted in the appearance of from 23 to 138 lymphocytes per cubic millimeter without a significant increase in the protein content. In three similar cases, however, the injection of 15,000 units was followed by moderate to severe headache, malaise, and, in one instance, nausea and vomiting. The spinal fluid twenty-four hours later showed from 500 to 4,300 cells, mostly polymorphonuclears, and an increased protein content. The reactions in patients with meningitis who received intraspinal injections likewise varied. Some had an increase in the symptoms of meningeal irritation accompanied by a persistent or increased pleocytosis in the spinal fluid, and others showed return of the spinal fluid essentially to normal while daily injections were still being continued.

#### PENICILLIN LEVELS IN PLEURAL FLUIDS

Most workers have not found significant concentrations of penicillin in pleural fluids during intramuscular therapy.<sup>12, 13</sup> After intrapleural injections of from 5,000 to 70,000 units in patients with empyema, it is absorbed rather slowly so that considerable amounts have been found in the fluid up to ninety-six hours. The amount found and its persistence apparently depends on the

amount injected and on the nature and size of the cavity.<sup>3, 12-17</sup> Transient pleural pain, fever, and pleocytosis may result from injections into the noninflamed pleura.<sup>14</sup> Rammelkamp and Keefer<sup>3</sup> could detect no penicillin in the blood serum after an intrapleural injection of 5,000 units in one patient. They found only the smallest measurable amounts after 10,000 units in a second patient and up to 0.03 unit per cubic centimeter of serum after 30,000 units in a third patient in which samples were taken up to about seven and one-half hours.\*

The present observations include (1) levels of penicillin in pleural fluid obtained after intramuscular injections alone, (2) serum penicillin levels after intrapleural injections when penicillin was not given by other routes, and (3)

TABLE II. CONCENTRATION OF PENICILLIN IN SERUM AFTER INTRAPLEURAL INJECTION

CASE	DOSE (UNITS)	INTERVAL (HOURS)	PENICILLIN IN SERUM (UNITS PER C.C.)†
1	50,000	1	0.06
		2	0.06
		3	0.06
		5	0.03
		12*	0.00
2	50,000	3	0.11
		4	0.03
		7*	0.00
3	50,000	1	0.00
		2	0.00
		3	0.00
		4	0.03
		5	0.03
4	20,000	8*	0.00
		1	0.00
		2	0.00
		3	0.00
		6	0.00
5	50,000	2	0.03
		4	0.03
		6	0.03
		8*	0.00
		3	0.06
6	50,000	5	0.06
		7	0.06
		9	0.03
		11*	0.00
		2	0.11
7	50,000	4	0.06
		6	0.03
		8*	0.00
		4	0.11
		5	0.03
8	50,000	4	0.11
9	50,000	5	0.03
10	50,000	1	0.22
		2	0.11
		4	0.03
		6	0.00
		8	0.03
11	60,000	1	0.11
		3	0.11
		6	0.11
12	60,000	1	0.03
		2	0.03
		4	0.03
		6*	0.00
13	50,000	$\frac{1}{2}$	0.22
		1	0.22
		2	0.11
14	40,000	4	0.22

Cases 6, 7, and 13 had serosanguineous effusions. The others all had empyema with thick purulent exudate, except Case 1, in which the fluid was serous in character.

\*0 in one or more subsequent specimens of serum.

†0 here means <0.03 unit which was the smallest amount measured.

\*Florey and Heatley<sup>22</sup> obtained bacteriostatic levels in the blood stream for twenty-four hours after 120,000 units and for forty-eight hours after 240,000 units injected intrapleurally.

pleural fluid levels after intrapleural injections with or without concurrent intramuscular therapy.

Concentrations of penicillin in pleural fluids from one to two and one-half hours after intramuscular injections were measured in five patients with empyema and in one with rheumatic effusion. A single 40,000 unit dose was used on one patient, while the others were under treatment with doses of 15,000 or 20,000 units every two or three hours. Levels ranging from 0.03 to 0.22 unit per cubic centimeter of pleural fluid were obtained. The level in the serum taken at the time of the aspiration was the same in one instance and from two to eight times higher in the others.

Serum levels in fourteen patients at various intervals after intrapleural injections of from 20,000 to 60,000 units of penicillin are shown in Table II. The intrapleural penicillin was given in concentrations of from 500 to 1,000 units per cubic centimeter in saline, and none of the patients were receiving penicillin by other routes at the time. Penicillin was not detected in the serum in the one patient (Case 4) who received 20,000 units. In another patient (Case 3) in whom 50,000 units were injected, minimum levels were observed in the serum at four and five hours, but none was detected before or later. In the remaining patients, concentrations ranging from 0.03 to 0.22 unit per cubic centimeter were found in the serum up to five or six hours after the intrapleural injection of from 40,000 to 60,000 units. In only two instances were detectable amounts found later: after eight hours in Case 10 and after nine hours in Case 6. These levels are comparable with those obtained in the serum of different individuals about two hours after an intramuscular injection of 15,000 units as shown in Fig. 1.

TABLE III. CONCENTRATION OF PENICILLIN IN PLEURAL FLUID AFTER INTRAPLEURAL INJECTION

CASE	INTRAPLEURAL DOSE (UNITS)	INTERVAL AFTER DOSE (HOURS)	PENICILLIN IN PLEURAL FLUID (UNITS PER C.C.)
1	50,000	24*	5.00
		48	0.11
2	50,000	24*	0.03
	50,000	24	0.45
3	50,000	24*	2.5
		72	0.03
	50,000	24	0.45
	50,000	48	2.5
10	50,000	24*	0.11
	50,000	24	0.11
11	60,000	24*	2.5
		144	0.00
	50,000	48	5.00
12	60,000	24*	40.00
13	40,000	24	0.11
15	50,000	18	2.5
	50,000	48	1.8
	50,000	48	0.06
	50,000	48	0.22
16	10,000	24	0.9
	10,000	24	1.8
	10,000	24	1.8
		216	0.00
17	30,000	24	0.22
18	40,000	24	10.00
19	20,000	24	5.00
20	30,000	24	1.8

\*These fluids were obtained after the injections listed on Table II.

Except in Case 20, in which there was a hydrothorax, all the fluids obtained before the injections were infected, thick, and purulent and were from patients with pneumococcal or streptococcal empyema.

Penicillin was given intramuscularly, usually 15,000 or 20,000 units every three hours, at the time these fluids were obtained, except Cases 2, 16, and 19, and before the aspirations indicated by the asterisks.

The concentration of penicillin in pleural fluids at various intervals after intrapleural injections of from 10,000 to 60,000 units varied considerably as shown in Table III. Intramuscular penicillin was being used in some and not in others. Even after the larger doses the concentrations in the pleural fluid obtained after twenty-four or forty-eight hours ranged from 0.03 to 40.0 units, most of them being between 0.11 and 5.0 unit per cubic centimeter. In some of the cases in which the concentrations in the pleural fluid were low, serum obtained at the same time showed higher levels. In one patient (Case 15), for example, serum levels obtained at the time of each of the last two fluids were 0.45 unit per cubic centimeter. In Case 10, the serum level each time was 0.22 unit per cubic centimeter.

Briefly, therefore, it would seem from the data presented that there is some diffusion from the blood stream into the pleural fluid as well as from the pleura into the blood, but it is quite variable in either case. Likewise, the concentrations found in the pleural fluid after the local instillation are erratic. This is probably due, at least in part, to the variable absorption from the pleural cavity. The possibility of loculation of infected fluids accounting for some of the wide discrepancies cannot be ruled out. There was no definite correlation between the specific gravity of the pleural fluid and the diffusion into it from the blood or from it into the blood. Furthermore, the concomitant intramuscular administration of penicillin did not seem to influence significantly the concentration in the pleural fluid after local instillation.

#### PENICILLIN LEVELS IN OTHER SEROUS CAVITIES

Very few observations have been reported on the diffusion in and out of body cavities other than the pleura and subarachnoid space. Small amounts have been measured in the serum for several hours after inoculation of 10,000 units into an infected knee joint.<sup>3</sup> This slow absorption from the synovial cavity has been suggested as a method of administering penicillin to obtain a prolonged systemic effect.<sup>18</sup> Penicillin has also been found in joint fluid during intravenous or intramuscular injections in a concentration about one-half of that found in the serum.<sup>6</sup> In infants and children, penicillin has been found in peritoneal fluid after intramuscular injection and also in the serum after intraperitoneal injections.<sup>10\*</sup> The present study includes a few observations on synovial, peritoneal, and pericardial fluids.

*Joint Fluids.*—Six specimens were obtained by aspiration of the knee joints in three patients under treatment with 15,000 or 20,000 units of penicillin intramuscularly every three hours. Two of the patients had purulent gonococcal arthritis and one had acute rheumatic fever. The fluids were obtained from one-half to two and one-half hours after an intramuscular injection. The levels were 0.03 unit per cubic centimeter in five specimens and 0.06 unit per cubic centimeter in the sixth. These levels were the same or lower than corresponding serum levels.

*Ascitic Fluid.*—Infected peritoneal fluid was obtained on four occasions from one patient with cirrhosis of the liver and ascites who had a complicating bacteremic staphylococcal infection and was receiving 20,000 units of penicillin intramuscularly every two hours. The level of penicillin in the peritoneal fluid was 0.03 on two occasions, 0.06 on one, and none was detected in the fourth.

\*Florey and Heatley<sup>3</sup> detected penicillin in the blood for thirteen hours in one patient and for twenty-four hours in another after injecting 120,000 units into an intact but infected knee joint. Greene and Altire-Werber<sup>10</sup> detected penicillin in the blood after intraperitoneal instillation.

One of the fluids showing 0.03 unit was obtained four hours after an intramuscular injection, and the serum had no demonstrable penicillin at that time. The sera obtained at the time of the two other positive fluids had the same concentration of penicillin as those fluids.

*Pericardial Fluid.*—Five specimens were available in two patients under treatment for purulent pericarditis. In one patient with streptococcal infection, 0.06 unit per cubic centimeter was found in the fluid aspirated during intramuscular therapy. In this case the fluids obtained twenty-four hours after each of two local injections of 20,000 units contained 2.5 and 5.0 units per cubic centimeter. The second patient had pneumococcal pericarditis and received two injections of 25,000 units each into the pericardial cavity after aspirations. The fluid obtained twenty-four hours later contained 20 units per cubic centimeter in one and 160 units per cubic centimeter in the other.

These findings indicate that penicillin diffuses into inflamed synovial, peritoneal, and pericardial cavities from the blood stream. From the pericardial cavity, as from the pleura and subarachnoid space, it seemed to diffuse out rather slowly in the two cases studied—at least high concentrations were still found twenty-four hours after the local injections.

#### SERUM LEVELS AFTER INHALATION OF PENICILLIN

The feasibility of utilizing penicillin in the form of an aerosol from a nebulized solution was pointed out by Bryson, Sansome, and Laskin.<sup>19</sup> These workers demonstrated penicillin in the lungs and urine of animals after inhalation. They were able to recover as much as 60 per cent of penicillin administered to cooperative human subjects who inhaled the aerosol directly from a nebulizer placed in the mouth. Clinical methods and apparatus for applying this procedure therapeutically were reported by Barach and his co-workers.<sup>20</sup> These authors demonstrated penicillin in the serum for one hour and occasionally longer after doses of from 40,000 to 100,000 units per cubic centimeter. They recovered from 10 to 20 per cent of the administered penicillin in the urine—most of this in the first hour.

The present observations were made during clinical trials of the methods described by Barach with some modifications by Dr. Maurice S. Segal, who also conducted the clinical studies. A total of eighty-one sera from seventeen patients under treatment with inhalations of from 20,000 to 30,000 units every two to four hours and from one normal subject after single inhalations of 20,000 or 25,000 units contained 1 c.c. of saline. Penicillin was not given by any other routes in these patients. The patients were being treated for acute or chronic pulmonary infections. The inhalations were usually completed within ten to twenty minutes. The frequency with which various levels were obtained at different intervals after the inhalations is summarized in Table IV. Significant

TABLE IV. PENICILLIN IN SERUM AFTER INHALATION

HOURS AFTER DOSE	PENICILLIN, UNITS PER C.C. OF SERUM					NUMBER OF SERA TESTED	PER CENT POSITIVE SERA*
	<.03	.03	.06	.11	.22		
½		1	2			3	100
1	13	7	4	1	1	26	50
2	16	6	1	2	1	26	38
3	19	1	1		1	22	14
4	4					4	0
Total	52	15	8	3	3	81	35

\*.03 unit per cubic centimeter or more.

The inhalations were given from nebulized solutions of from 20,000 to 30,000 units of commercial penicillin contained in 1 c.c. of physiologic saline every two to four hours and required from ten to twenty minutes to complete each dose.



concentrations were found in the serum for only a short time in most instances. In a few, however, penicillin was detected for as long as two hours. While most of the serum levels were low, occasional ones were comparable to those obtained two hours after intramuscular injections of 20,000 units in similar subjects. The highest and most sustained levels were obtained in two patients with pneumonia and in one with a lung abscess. Of the six sera showing levels of 0.11 unit or more per cubic centimeter, five were from a single patient who had a large lung abscess.

It should be borne in mind that these observations were made chiefly in sick patients, not all of whom could cooperate properly. Furthermore, the types of apparatus were being frequently changed and improved and relatively small doses were used. Higher levels can be expected for longer periods and more regularly with larger doses and improved apparatus in more cooperative patients.

#### DISCUSSION

It was the primary purpose of this paper to present observations on penicillin levels in serum and in some of the body fluids which are commonly infected with susceptible organisms. The data were obtained almost entirely on patients while they were under treatment. The method used is subject to considerable error, but it is adequate for these purposes since it was not intended here to offer any accurate measures of the absorption and fate of penicillin in the body.

Similar observations have been made with a different method by Selbie and co-workers<sup>21</sup> and with comparable results.

The concentrations of penicillin which destroy the common bacteria encountered in "medical" infections were studied in this laboratory by a very similar method<sup>1</sup> so that the results are comparable. They indicate that the smallest concentration of penicillin which was measured in the present study, namely, 0.03 unit per cubic centimeter, is adequate to sterilize actively growing cultures of almost all strains of gonococcus, group A hemolytic streptococcus, and pneumococcus and most strains of alpha hemolytic streptococcus, about half of the strains of meningococcus, and a somewhat smaller proportion of strains of pathogenic staphylococci. Undoubtedly the accessibility of the penicillin to the organism and many other factors, most of which are still unknown, also come into play and account for some of the discrepancies between *in vitro* and *in vivo* results.

The serum levels of penicillin obtained after various doses and intervals during systemic administration indicate that for any given dosage only a range of levels rather than any specific values can be predicted. In general, higher levels are attained and sustained with the larger and more frequent doses. The levels are not directly proportional to the increase in the dose except possibly in any given individual under constant intravenous therapy.<sup>22</sup> The results in patients with cardiac or renal insufficiency indicate that the volume of urinary output and the state of the kidney function may materially affect the height and persistence of penicillin levels after any given dose. Such patients usually attain and maintain any given level with appreciably smaller doses given at longer intervals than do persons with normal renal function and those who excrete larger volumes of urine.

The problem of whether or not topical injections are necessary in cases of meningitis or of infections of serous cavities or in walled-off focal abscesses will be answered more directly on the basis of clinical results than by any

analysis of data such as those given in this paper. The present observations and similar studies by others indicate that there is some diffusion into all serous cavities but that this is erratic and usually is only slight. Clinically, it is already recognized that these infections, after they have become established, do not often respond to systemic treatment alone in spite of the fact that bacteriostatic concentrations for the offending organisms can often be demonstrated in the exudate.

In cases of meningitis there are differences expressed in the literature both as to the penetration of penicillin into the cerebrospinal fluid and as to the necessity for having such penetration. Small amounts of penicillin have been found by others in individual cases particularly after massive systemic doses.<sup>23</sup> This has not been our experience nor that of others who have used larger doses in a greater number of cases. Some cases of bacterial meningitis have been reported as cured without resort to intrathecal injections of penicillin,<sup>24</sup> but these cases are not entirely convincing since few, if any, of these patients had received systemic penicillin as the only specific therapy. On the other hand, the experience in this clinic and in many others would indicate that, not only is intraspinal therapy necessary, but it is further necessary in some instances to resort to injections into the cerebral subarachnoid space or even into the ventricle<sup>9</sup> for complete recovery.

The necessity for systemic treatment when penicillin injections are given into infected body cavities will depend on the circumstances in each case. Some absorption takes place from all the cavities, but this, too, is erratic and effective levels are sustained in the circulation for variable periods. Some workers have found the poorest absorption from thick-walled cavities and from abscesses which are well walled off.<sup>21</sup> Obviously, therefore, if there is evidence of infection of tissues away from the infected cavity into which the penicillin is being injected, systemic treatment is definitely indicated. The absorption from the cavity may, however, be adequate to protect against spread of the infection from the site of the injection as a result of the procedure. It is possible that diffusion out of cavities is delayed in some cases when systemic therapy is given at the same time<sup>10</sup> and that may be a reason for using it. Evidence for this, however, is scant.

#### SUMMARY AND CONCLUSIONS

In this paper are presented the results of determinations of penicillin levels in serum and in some body fluids of patients under treatment with various doses of penicillin given by different routes. When compared with the results of tests for sensitivity of the offending organism, such information helps to indicate in a very general way the results to be expected from therapy in various types of cases.

During continuous intravenous or intramuscular administration, fairly constant levels are maintained in the serum of any given patient, but the levels may vary in different patients on the same dose.

During intermittent intramuscular injections a range of serum levels but not exact values can be predicted for the interval between doses on any given dosage schedule. Larger doses result in serum levels which are sustained longer and at higher values than smaller ones.

During intramuscular penicillin therapy it was not possible to detect penicillin in the cerebrospinal fluid, and the levels in pleural, peritoneal, and synovial fluids were erratic and usually lower than the serum levels.

Diffusion from local injections of penicillin into body cavities out into the serum is quite erratic, but adequate concentrations usually remain in cerebrospinal, pleural, and pericardial fluids for twenty-four hours and sometimes for forty-eight hours or longer after local injections of large amounts.

After inhalation of penicillin from nebulized solutions, small amounts may be detected in the serum for short periods but rarely for as long as two hours.

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## RH AGGLUTINATION IN VARIOUS MEDIA WITH PARTICULAR REFERENCE TO THE VALUE OF ALBUMIN

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THE technique for the detection of anti-Rh antibodies has undergone several variations within the past few years. The first method in use, the "modified tube compatibility test" (incubation at 37° C. for one hour of unknown serum with 2 per cent suspension of Rh-positive red cells in saline), was found to be inadequate since from 40 to 50 per cent of the subjects in whom sensitization was later confirmed, by the birth of an erythroblastotic child or by a hemolytic transfusion reaction, showed negative tests for anti-Rh agglutinins. An explanation for such failures was found independently by Wiener and by Race. Race<sup>1</sup> found an additional evidence of Rh sensitization which he called an "incomplete antibody" that did combine with the antigen (the Rh-positive red cell) but failed to cause agglutination (in the saline suspension of cells in the test tube). At the same time Wiener<sup>2</sup> recognized the same phenomenon, that is, failure of agglutination of the Rh-positive cells by the serum of some sensitized individuals. He demonstrated it as follows: to the usual mixture of 2 per cent suspension of Rh-positive cells in saline and unknown serum from the sensitized person, he added one drop of an anti-Rh serum known to be capable of agglutinating the test cells. The difference in degree of agglutination of this system, after incubation at 37° C and centrifugation, from the expected gross or 4 plus agglutination was the degree of inhibition or "blocking" caused by the unknown serum. Diamond and Abelson<sup>3</sup> have shown that the combination of these two anti-Rh tests (that is, ordinary agglutination plus "blocking" test) is much more satisfactory than the first alone, in that it demonstrated the presence of Rh antibody in 92 per cent of their cases of sensitization.

With the development of the rapid slide technique, Diamond and Abelson<sup>4</sup> proved that recognition of both agglutinin and inhibitor, or "blocking," antibodies was possible in almost 100 per cent of the individuals in whom sensitization had occurred. They found, however, that their test required Rh-positive cells in the form of whole blood and that the removal or marked dilution of the plasma of such blood (by washing repeatedly with saline) inhibited the reaction on the slide. As a result of these observations, they concluded<sup>5</sup> that plasma provided a medium in which Rh inhibitor, or "blocking," antibodies could cause agglutination or clumping of Rh-positive cells, and that absence or great dilution of this medium reduced the effect of the inhibitor antibody on test cells to merely a "blocking" action not demonstrable by any recognizable clumping. Wiener<sup>6</sup> suggested that this phenomenon might be due to a colloidal effect of the conglutinin of plasma. He named the reaction "conglutination" to distinguish it from agglutination in ordinary saline diluent. He confirmed that the results

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The use of albumin for enhancement of Rh agglutinins was suggested by the workers in the Department of Physical Chemistry, Harvard Medical School, and has been reported by Cameron and Diamond in the *Journal of Clinical Investigation* (in press).

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of the slide test could be reproduced in the test tube when plasma was substituted for saline as a diluent for the Rh-positive test cells.

Further studies have now been carried out on the effects of plasma in the testing of sera, which, though produced by sensitized individuals, failed to cause agglutination of Rh-positive red cells in saline diluent. Parallel titrations of a number of such known "blocked" sera, diluted serially in saline and in plasma, were set up, using twice-washed Rh-positive test cells likewise suspended in saline and in plasma. The technique was as follows: one drop of serum of each of the various dilutions was added to two drops of a 2 per cent suspension of the test cells in their respective diluent. The mixture was incubated at 37° C. for one hour, centrifuged at 500 r.p.m. for one minute, and read macroscopically with the aid of a hand lens. Weak and negative reactions were checked microscopically under low power. The titrations in the saline system showed no agglutination in any dilution, as previously had been demonstrated in these "blocked" sera. The plasma systems, however, showed strong clumping of the test cells. A typical result is shown in Table I. The

TABLE I. TITRATION OF ANTI-RH BLOCKING ANTIBODY IN SALINE AND IN PLASMA MEDIA (WASHED TEST CELLS, GROUP O, RH<sub>+</sub>; BLOCKING ANTIBODY, ANTI-RH<sub>0</sub>)

SERUM DILUTIONS	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
2 per cent cells in saline (serum diluted with saline)	0	0	0	0	0	0	0	0	0
2 per cent cells in plasma (serum diluted with plasma)	Solid	Solid	++++	++++	+++	++	+	+	Trace

plasma diluent in this case either has supplied some factor by virtue of which the "blocking" effect of the antibody, as shown by the absence of reaction in saline, has been removed or has provided a more favorable medium than water, allowing the antibody-antigen reaction to become manifest by the clumping of the test cells to an end point of 1:256 with this particular serum. Thus the total content of Rh antibody is taken as 256, all of which is inhibitor, or "blocking," antibody. Similar experiments with sera containing both agglutinin and "blocking" antibody demonstrate that the saline diluent system, or, for that matter, any suitable salt in water, shows agglutination to one end point (often associated with a prozone phenomenon), whereas the plasma system has an end point at a much higher dilution. In such a case the ordinary agglutinin titer may be designated as the end point of the saline titration and the total antibody titer as the end point of the plasma titration, the difference between them being due to the "blocking" antibody present. A protocol of one of these experiments is shown in Table II. In this serum the ordinary agglutinin titer in saline is 1:8 and the total antibody titer, 1:512. The difference between these two levels is due to inhibitor antibody unmasked in the plasma diluent. Sera

TABLE II. TITRATION OF ANTI-RH BLOCKING-AGGLUTININ ANTIBODY IN SALINE AND IN PLASMA MEDIA (WASHED TEST CELLS, GROUP O, RH<sub>+</sub>; ANTIBODY-AGGLUTININ, ANTI-RH'; AND BLOCKING ANTI-RH<sub>0</sub>)

SERUM DILUTIONS	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
2 per cent cells in saline (serum diluted with saline)	++	+++	++	Trace	0	0	0	0	0	0	0
2 per cent cells in plasma (serum diluted with plasma)	Solid	Solid	Solid	Solid	++++	+++	++	++	+	Trace	0

containing known agglutinin but no "blocking" antibody have been tested in this way and have shown the same end point of agglutination in both saline and plasma systems, indicating that all antibody present is demonstrated in the saline and that no further agglutination can be demonstrated by the use of plasma.

The use of plasma as a diluent for test cells, therefore, provides a method by which anti-Rh inhibitor antibody may be demonstrated in a test tube, the counterpart of the slide test of Diamond and Abelson. The exact nature of the mechanism by which plasma permits the demonstration of "blocking" antibody by means of agglutination will not be discussed here, although a colloid-like property, as suggested by Wiener, is undoubtedly one possibility. That complement is not responsible has been proved beyond doubt by the reproduction of the results described, using inactivated antibody serum and diluent plasma (heated at 60° C. for one hour). Dilution of the plasma with saline weakens or prevents the reaction of agglutination when the proportion of saline diluent to total contents of the tube reaches approximately one-third.\* Once agglutination has occurred, however, the further addition of saline has no injurious effect.

Certain obstacles arose to the adoption of plasma as a diluent for test cells in anti-Rh determinations. Foremost among these was the unpredictable occurrence of rouleaux formation in the test cells suspended in plasma; this appeared to be distinct from the clumping due to antibody-antigen reaction but nevertheless made gross and microscopic readings, especially of titration end points, often difficult to recognize. The cause of rouleaux formation could not be determined since it occurred in both Rh-positive and Rh-negative diluent plasmas, whether that removed from the cells themselves or that obtained from another source. In addition, the routine use of plasma demands a constant source of blood of Group AB (in order to avoid isohemagglutinin reactions if A or B test cells are used) and of the Rh-negative type (because the possibility of soluble Rh substance in plasma from an Rh-positive donor causing reduction or neutralization of Rh antibody in the serum being tested must be avoided). These objections to the use of plasma were of sufficient magnitude to make it desirable to seek a better suspension medium for test cells.

The following materials were tried as diluents for red cells: (1) albumin, human crystalline, (2) albumin, bovine crystalline, (3) gelatin, (4) methyl cellulose, (5) polyvinyl alcohol, and (6) dextrose (only because of its viscous nature). The anti-Rh sera for testing were selected because they had no demonstrable free agglutinin (against a saline suspension of test cells) but did have a high titer of "blocking," or inhibitor, antibody (demonstrated by the tube blocking test). The test cells consisted of twice-washed Group O Rh<sub>1</sub>, Rh<sub>2</sub>, and Rh-negative cells in 2 per cent suspension in each of the diluents. One drop of undiluted serum was placed in each of a number of small test tubes; two drops of the test cell suspensions in their respective diluents were added to each tube. The mixture was then shaken, incubated in a water bath at 37° C. for one hour, centrifuged at 500 r.p.m. for one minute, and read macroscopically. Negative reactions were confirmed microscopically. Parallel tests, using a saline and a plasma diluent, also were set up at the same time. The results with Rh<sub>1</sub> cells only (Rh<sub>2</sub> cells reacted in a similar way and, therefore, are not included in this protocol) are shown in Table III.

\*This appears to be due not to the saline content, but to the water content. However, some electrolyte must be used in order to prevent hemolysis of cells.

TABLE III. THE ACTION OF ANTI-RH BLOCKING ANTIBODY ON RH + CELLS SUSPENDED IN VARIOUS MEDIA  
(BLOCKING ANTIBODY, ANTI-RH<sub>0</sub>)

DILUENT MEDIUM FOR TEST CELLS	WASHED TEST CELLS	
	GROUP O, Rh <sub>+</sub>	GROUP O, Rh NEGATIVE
Saline	0	0
Plasma	+++	0
Human albumin (25%)	+++±	0
Bovine albumin (30%)	++++	0
Gelatin	+++±*	++±*
Methyl cellulose	+++*	+++*
Dextrose	0	0
Polyvinyl alcohol	0	0

\*Matting and agglutination—cell damage.

The albumins, human and bovine, as suspension media for Rh-positive cells not only permitted clumping with "blocked" sera equal to that obtained with plasma, but in addition gave firmer and more distinct aggregations. Furthermore, the tendency to rouleaux formation was never encountered in the use of these materials. Reactions in bovine and in human albumin were equally satisfactory; as a result, the former was used in all of the succeeding experiments because of its greater availability and lower cost. Our experience with the use of albumin as a diluent has indicated that its greater density requires slightly more prolonged centrifugation in order to produce a satisfactory cell button at the bottom of the tube; 500 r.p.m. for two minutes has been found adequate.

No effect whatever was demonstrated by use of dextrose or polyvinyl alcohol. Gelatin and methyl cellulose caused a matting and clumping of Rh-positive and Rh-negative cells alike, although the clumping in 5 per cent gelatin was rather more intense with Rh-positive cells than with Rh-negative cells, which might be significant if other conditions such as pH, for example, were controlled. This substance, however, was extremely difficult to handle in view of its tendency to solidify at room temperature.

Attention was given next to the problems of optimum concentration of albumin, the effects of dilution upon its action with "blocked" anti-Rh sera and, in addition, the optimum concentration of test cells required. Dilutions of 30, 20, 15, 10, 5, and 2 per cent bovine albumin were made up. The upper level of 30 per cent was chosen because this is the maximum concentration of the commercial product available and above this the viscosity of the medium becomes objectionable. The technique of tube testing was that described plus the additional centrifuging. A typical result of these experiments is given in Table IV. It is apparent that albumin at a concentration of 10 per cent fails

TABLE IV. THE EFFECT OF VARYING CONCENTRATIONS OF ALBUMIN ON THE RH BLOCKING ANTIBODY-ANTIGEN REACTION  
(BLOCKING ANTIBODY, ANTI-RH<sub>0</sub>)

CONCENTRATION OF BOVINE ALBUMIN DILUENT FOR TEST CELLS (%)	WASHED TEST CELLS		
	GROUP O, Rh <sub>+</sub>	GROUP O, Rh <sub>+</sub>	GROUP O, Rh NEGATIVE
30	++++	++++	0
20	+++±	++++	0
15	++±	++++	0
10	0	++±	0
5	0	0	0
2	0	0	0

to permit clumping of Rh<sub>1</sub> test cells in this "blocking" serum, while a concentration of 15 per cent gives positive, though somewhat weak, reactions. Twenty per cent albumin, however, yields uniformly good reactions, and further experiments with many such "blocked" sera have corroborated this finding. When a cell diluent of 20 per cent albumin is used with this technique, it will be seen that the albumin content of the whole system in the tube falls to 13 per cent, ignoring the content of the original test serum. In repeated testing, this level of albumin concentration was found to be the critical point, below which reactions were weak or absent and above which they were uniformly strong.\* The determination of optimum concentration of test cells in albumin was decided by the trial of various concentrations from 2 to 50 per cent of washed cells in 20 per cent albumin. All of these concentrations demonstrated equally good agglutination response to the antibody, although the 2 per cent suspension proved easier to prepare, handle, and read. For these reasons it was selected for use in the succeeding experiments.

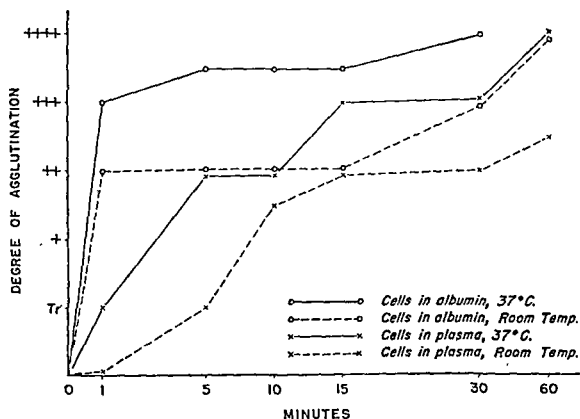


Fig. 1.—The rate of reaction of Rh-positive red cells in albumin and in plasma with Rh antibody of the "blocking" type

The rate at which the antibody-antigen reaction takes place in plasma and in albumin was considered next. Two per cent suspensions of washed Rh<sub>1</sub> cells in plasma and in 20 per cent albumin were set up; one series of each was studied at 37° C., another at room temperature. The degree of reaction was read at one, five, ten, fifteen, thirty, and sixty minutes. The usual technique of incubation and centrifugation was used. The results of this experiment are shown in Fig. 1. Agglutination in the incubated albumin system was macroscopically visible in one minute, reaching completion between fifteen and thirty minutes. The albumin system at room temperature and the incubated plasma system showed similar rates of agglutination, reaching the stage of maximum agglutination between thirty and sixty minutes; the reaction in the plasma system at room temperature was still incomplete at sixty minutes. For routine technique, from

\*The addition of albumin does not merely replace a deficit of albumin in the "blocking" serum, since albumin-globulin ratios in agglutinating and in "blocking" Rh antisera are within the normal range.



five to ten minutes in albumin and from fifteen to thirty minutes in plasma, at 37° C., yield valid and unmistakable results.

The effect of varying salt concentrations in the diluent media also was studied. Elliott<sup>6</sup> reported that anti-A and anti-B agglutination are enhanced by hypertonic sodium chloride concentrations in the test tube. Accordingly, Rh-positive cell suspensions were made up in three varieties of bovine albumin, hypotonic, isotonic, and hypertonic, and tested against a known "blocking" antibody. The hypotonic albumin system caused some swelling and hemolysis of test cells, and in addition the agglutinates were soft and difficult to read. On the other hand, the hypertonic and isotonic systems gave completely satisfactory results, the former causing slightly better agglutination than the latter.

A further experiment demonstrating that albumin is a satisfactory medium for the agglutination of Rh-positive cells by the inhibitor type of Rh antibody was carried out on a suggestion by Dr. Neva M. Abelson. Two drops of 2 per cent suspension of Rh-positive test cells in saline were "blocked" by the addition of one drop of antibody of a known inhibitor type. (Complete "blocking" was demonstrated by the tube method). The saline supernate was then withdrawn and replaced by two drops of 20 per cent bovine albumin. The mixture was shaken, allowed to stand at room temperature for five minutes, then centrifuged at 500 r.p.m. for two minutes, and read. Complete agglutination of the previously blocked cells had taken place, indicating that the presence of albumin had brought about a change in the test cells by which they were able to form agglutinates. This finding suggests a practical modification of the ordinary anti-Rh tests carried out in a tube with a saline suspension of test cells—if no agglutination of the saline suspension of test cells occurs (the reaction previously reported as negative), the supernatant saline is removed and replaced by albumin, the mixture allowed to stand at room temperature for five minutes, then it is re-centrifuged. The formation of agglutination in this new albumin system will prove the presence of inhibitor antibody in the unknown serum. Such a technique avoids the need for carrying out the tube "blocking" test, which requires the addition of another serum. Also it is economical in the amount of albumin used.

Having demonstrated the satisfactory elimination of "blocking" effect of Rh inhibitor antibody by the use of 2 per cent suspension of washed test cells in a diluent medium of 20 per cent albumin (human or bovine) of isotonic or slightly hypertonic salt concentration, a group of twenty Rh anti-sera, all containing inhibitor antibody, and some containing ordinary or early anti-Rh agglutinin as well, was studied by this technique. Each serum was tested with a 2 per cent suspension of washed Rh<sub>1</sub>, Rh<sub>2</sub>, and Rh-negative test cells in saline, plasma, and 20 per cent bovine albumin, respectively. In some cases additional tests were done with Rh' and Rh'' cells. The results showed without exception that albumin and plasma diluents demonstrated, by agglutination of the test cells, the presence of antibody where it failed to react in the presence of saline diluent. Furthermore, the albumin suspensions gave stronger reactions than did plasma and were completely free from any rouleaux-like formation so frequently encountered in plasma. No reactions were seen which were not compatible with Wiener's classification of the Rh series.<sup>7</sup> Interpretation of the combined reactions of cell suspensions using red cells of different subtypes, in saline and in albumin made it possible to determine in every case the specificity of the inhibitor antibody and of the simple agglutinin (if present). In Table V is demonstrated a typical experiment by which the type of "blocking" antibody

TABLE V. THE DETERMINATION OF THE SPECIFICITY OF RH ANTIBODY (BLOCKING AND AGGLUTININ) BY THE REACTIONS OF TEST CELLS SUSPENDED IN SALINE AND IN ALBUMIN

(ANTIBODY-AGGLUTININ, ANTI-RH'; AND BLOCKING, ANTI-RH<sub>0</sub>)

	WASHED TEST CELLS			
	Rh <sub>1</sub>	Rh <sub>2</sub>	Rh'	Rh NEGATIVE
2 per cent cells in saline	++++	0	++++	0
2 per cent cells in albumin	Solid	++++	Solid	0

may be specified. This serum shows agglutinin of type anti-Rh', since agglutination Rh' and Rh<sub>1</sub> cells occurs in the saline suspension. However, agglutination of Rh<sub>2</sub> cells in the albumin system, but not in the saline, is evidence of the presence of a "blocking" antibody of specificity anti-Rh<sub>0</sub>. This serum, therefore, is of the variety anti-Rh<sub>0</sub>' (87 per cent) with inhibitor of the Rh<sub>0</sub> type. Such a serum is commonly used in routine laboratory testing (in saline suspension) as the 70 per cent or anti-Rh' serum. "Blocking" antibodies in the group of sera tested were predominantly of the anti-Rh<sub>0</sub> type, although two cases demonstrating inhibitor antibody of both Rh<sub>0</sub> and Rh' specificity were encountered.

Titration of the three combinations of Rh antibody, i.e., simple agglutinin, combined "blocking" and agglutinin, and pure "blocking" antibody were carried out, the diluent media for test cells and antibody being saline and 20 per cent bovine albumin in each case. The results are shown in Table VI.

TABLE VI. TITRATION OF RH ANTIBODY OF THE VARIOUS TYPES WITH RH-POSITIVE CELLS, IN SALINE AND IN ALBUMIN SYSTEMS

A. Agglutinin Only (WASHED TEST CELLS, GROUP O, RH <sub>1</sub> ; ANTIBODY-AGGLUTININ, ANTI-RH', ONLY)												
SERUM DILUTIONS	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
2% cells in saline (serum diluted in saline)	Solid	++++	++++	++++	+++	++	Trace	0	0			
2% cells in albumin (serum diluted in albumin)	Solid	Solid	++++	++++	++++	++	++	Trace	0			
B. Agglutinin and Blocking (WASHED TEST CELLS, GROUP O, RH <sub>1</sub> ; ANTIBODY-AGGLUTININ, ANTI-RH'; AND BLOCKING, ANTI-RH <sub>0</sub> )												
2% cells in saline (serum diluted in saline)	++	+++	++	Trace	0	0	0	0	0	0	0	0
2% cells in albumin (serum diluted in albumin)	Solid	Solid	Solid	Solid	++++	++++	++++	++++	++	+	Trace	0
C. Blocking Only (WASHED TEST CELLS, GROUP O, RH <sub>1</sub> ; BLOCKING ANTIBODY, ANTI-RH <sub>0</sub> )												
2% cells in saline (serum diluted in saline)	0	0	0	0	0	0	0	0	0			
2% cells in albumin (serum diluted in albumin)	Solid	Solid	++++	++++	++++	+++	++	Trace	0			

From these findings it may be concluded that Rh-positive test cells in saline and in albumin diluents give the same type of reaction with the simple or early agglutinating antibodies; in the presence of inhibitor antibody alone the test cells in saline are "blocked," whereas in albumin they are agglutinated. When both types of antibody are present, their respective titers are indicated by an end point of agglutination in the saline system different from that in the albumin system. By such parallel titrations it is possible to determine the relative amounts of agglutinin and/or inhibitor antibody with considerable ac-

During these experiments a number of animal anti-Rh sera, produced by repeated injection of rhesus cells into guinea pigs, were titrated by the parallel saline and albumin medium method. One of these sera, previously rather unsatisfactory for routine use and readable only by the appearance of the cell button, showed a significant amount of inhibitor antibody, giving a markedly stronger reaction in albumin than in saline. A protocol of this experiment is shown in Table VII. It will be noted that the saline series shows a "prozone" effect which in our experience may be indicative of the presence of inhibitor antibody. This finding may explain the difficulties that have been encountered in the use of immune anti-Rh sera of animal origin. Such animal sera used with cells suspended in albumin may now yield entirely satisfactory results.

TABLE VII. COMPARATIVE TITRATIONS OF IMMUNE GUINEA PIG ANTI-RH ANTIBODY IN SALINE AND IN ALBUMIN MEDIA

(WASHED TEST CELLS, GROUP O, Rh<sub>2</sub>; ANTIBODY, IMMUNE GUINEA PIG SERUM)

DILUTIONS OF SERUM	1:1	1:2	1:4	1:8	1:16	1:32	1:64
2% cells in saline (serum diluted in saline)	Trace	+	±	+	Trace	0	0
2% cells in albumin (serum diluted in albumin)	Solid	++++	++++	++	±	Trace	0

The following presents an interesting case illustrative of the value of albumin in permitting agglutination of Rh-positive cells by anti-Rh inhibitor antibody when saline suspensions of test cells had repeatedly yielded negative results.

The patient, a woman 60 years of age, had lost her last three infants with jaundice, anemia, and edema, the last (stillbirth) being thirty years previously. No further pregnancies or transfusions had occurred in the intervening years. Because of the particular interest of the patient and her physicians in proving serologically that Rh incompatibility had caused erythroblastosis and death of the infants many years ago, blood studies were carried out recently on several occasions. The patient's red cells were Rh negative, the serum showed no anti-Rh agglutinins when tested repeatedly against all subtypes of Rh-positive cells suspended in saline solutions. The tube test failed to identify "blocking" antibodies. The slide test, however, was positive, yielding a slow ± (1½ plus) reaction. Tests of the serum with washed Rh<sub>1</sub> and Rh<sub>2</sub> cells suspended in albumin showed definite 4 plus agglutination, and titration of the serum, diluted serially in albumin and tested against Rh<sub>2</sub> cells in albumin, showed an Rh antibody titer of 1:8, which was "blocking" antibody since a parallel titration in the saline diluent showed no reaction. The specificity of the inhibitor or "blocking" antibody was anti-Rh<sub>0</sub>. This case demonstrates the sensitivity and certainty of results achieved by these newer testing methods for detection of Rh immunization. It also proves the long duration of permanence of such antibodies ("blocking" type); in this instance, thirty years post partum.

#### SUMMARY

The experiments described have followed a program of study of the action of anti-Rh antibodies, especially those designated as "blocking" or inhibitor antibodies, and their behavior toward Rh-positive cells suspended in various media. It has been recognized for a considerable time that certain types of Rh antibody, although known to be present, cannot be demonstrated readily by the tube agglutination of test cells suspended in saline, thus making the ordinary anti-Rh determination sometimes difficult and often inconclusive. The demon-

stration by Diamond and Abelson that these "blocking" sera would cause agglutination, on a slide, of Rh-positive whole blood provided the direction in which the experiments were aimed.

As reported elsewhere, in experiments involving the sensitization of Rh-negative subjects and in studies of sensitization of Rh-negative women by repeated pregnancies, it has been found that the simple anti-Rh agglutinin which produces clumping of Rh-positive cells in saline diluent is the first to appear and may therefore be called the early agglutinin or immature antibody. After repeated stimulation of experimental subjects or following multiple pregnancies in some women, inhibitor or "blocking" antibody appears. This may therefore be called the late, mature, or complete antibody.

Anti-Rh antibody, whether of the early, immature type or of the mature, late ("blocking") type, has been shown to react specifically with Rh-positive test cells in the test tube by the formation of clumps when a 2 per cent suspension of such cells in 20 per cent albumin is used. The degree of reaction, as manifest by the solidity of agglutinates, is considerably enhanced over that which occurs in saline suspensions, and in addition no inhibition of agglutination occurs when "blocking" or late antibody is present. Furthermore, serial dilution titrations of Rh antibodies regardless of the type (agglutinin, combined agglutinin and "blocking," pure "blocking"), using 20 per cent bovine albumin in one series and saline in another as diluent for both antibody and test cells, will demonstrate the relative amounts of agglutinin and of "blocking" antibody.

A modification of the tube anti-Rh test, involving the use of albumin in eliminating "blocking" effect, is proposed. This modification consists of the removal of supernatant saline in those anti-Rh tests which appear to be negative and replacement of this saline supernate by an equal amount of 20 per cent albumin. If any Rh antibody is present, agglutination of the previously non-reactive test cells will occur at room temperature within a few minutes.

The different behavior of Rh-positive cells of the various subtypes (Rh, Rh', Rh'') in saline and in albumin media toward Rh antibodies of the inhibitor type provides a method by which the specificity of such "blocking" antibodies may readily be determined. This technique is appreciably simpler and the results more clear-cut than the "blocking test" of Wiener.

No nonspecific effects of albumin have been encountered nor any behavior of Rh antibody with test cells not in keeping with the present classification of the Rh series. However, it should be kept in mind that if albumin is unavailable, plasma or serum which has been tested as free of nonspecific effects may be used for the tests described here.

As a result of these studies, as well as of observations during sensitization on the time relationship of the appearance of the simple agglutinating antibodies (active in saline suspensions of Rh-positive cells) and the "blocking" antibodies (inactive in saline but active in albumin), it seems advisable to consider more descriptive names for these two forms of the Rh antibody. The terms "early, or immature, antibody" would seem to apply to that causing agglutination in saline suspensions, whereas "late, or mature, antibody" is more descriptive of the "blocking" antibody which is recognizable only in albumin or plasma media and is associated with more severe or prolonged sensitization.

The use of albumin as a suspension medium for red cells has proved the potency of many sera previously discarded as containing little or no agglutinin and useless for laboratory determination of the Rh factor. Such sera may now become useful. This applies to some of the immune sera produced in guinea

pigs and other animals. A method for making many discarded sera valuable for routine use, even with saline suspension of red cells, is described in another communication.

Finally, as proved repeatedly in this laboratory and as described herewith in an illustrative case, an albumin suspension medium for test cells can readily detect weak agglutinins of the mature or "blocking" type in individuals who have been sensitized many years previously.

#### CONCLUSIONS

A series of experiments demonstrating the action of Rh antibodies on Rh-positive cells suspended in various media has been described. Inhibition of agglutination of Rh-positive cells by the so-called "blocking" antibody does not occur in the presence of plasma or albumin. Because of its freedom from non-specific effects, albumin has proved the most satisfactory suspension medium for test cells. Bovine acts similarly to human albumin and is, at present, considerably cheaper. It is important that the albumin content be added in a concentration above 13 per cent. The evaluation of early or immature antibody and of late or mature antibody is possible by comparative titrations in saline and in albumin systems.

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### A CLINICAL EVALUATION OF THE COPPER SULFATE METHOD FOR MEASURING SPECIFIC GRAVITIES OF WHOLE BLOOD AND PLASMA

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A SIMPLE method for determining hemoglobin, hematocrit, and plasma protein values has recently been proposed by Phillips and co-workers.<sup>1, 2</sup> These values are derived from the specific gravities of whole blood and of plasma or serum with the assistance of line charts. The specific gravities are determined by the use of a series of copper sulfate solutions of graduated density which are readily prepared from a stock standard solution. The whole process requires no complicated apparatus and is easily adaptable to either laboratory or field work. The present study was undertaken to test the clinical applicability of this newly devised technique in a general hospital.

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*Number of Tests for Which the Standard Copper Sulfate Solutions May Be Used.*—Solutions of copper sulfate were prepared as directed by Phillips and associates in volumes of 50 c.c. with specific gravities ranging from 1.008 to 1.075, graduated in units of 0.002. The specific gravities within the range of 1.020 to 1.030 were measured by the gradient tube method of Linderström-Lang<sup>3</sup> as applied by Lowty and Hastings<sup>4</sup> and showed a maximum deviation from the expected values of +0.0004 (Table I).

TABLE I. DETERMINATION, IN GRADIENT TUBE, OF THE SPECIFIC GRAVITIES OF COPPER SULFATE SOLUTIONS, BEFORE AND AFTER ADDITION OF 50 DROPS OF PLASMA TO 50 C.C. OF EACH SOLUTION

EXPECTED SPECIFIC GRAVITY	SPECIFIC GRAVITY FOUND BY GRADIENT TUBE		CHANGE CAUSED BY ADDITION OF PLASMA
	UNUSED SOLUTION	SOLUTION AFTER RECEIVING PLASMA	
1.0200	1.0204	1.0199	-0.0005
1.0220	1.0222	1.0219	-0.0003
1.0240	1.0242	1.0235	-0.0007
1.0260	1.0264	1.0256	-0.0008
1.0280	1.0283	1.0281	-0.0004
1.0300	1.0301	1.0299	-0.0002
Average change			-0.0005

A duplicate set of solutions, to each of which 50 drops of plasma had been added, was similarly checked by the gradient tube, and the average lowering of the specific gravity of the copper sulfate solutions, caused by adding one drop of plasma per cubic centimeter of solution, was found to be 0.0005 (Table I). Under ordinary conditions each set of 50 c.c. copper sulfate solutions can be used for determining the gravities of whole blood and plasma of about seventy-five blood samples without adding more than 50 drops to any one bottle.

*Constancy of Results.*—The first requisite of a quantitative method is that it yield results which can be duplicated within definite limits of error by different analysts. The copper sulfate method was tested in this respect by comparing duplicate specific gravities of the same specimens of whole blood and plasma determined by two observers (Table II).

Each specimen of blood in this work was drawn into a dry syringe. The greater part of the blood was delivered from the syringe into a hematocrit tube containing heparin. The excess blood left in the syringe was used at once to determine the gravity of the whole blood. The plasma separated in the centrifuged hematocrit tube was drawn into another dry syringe and used to determine plasma gravity.

From the gravities, plasma protein and blood hemoglobin concentrations were calculated by the following equations,<sup>2</sup> in which  $G_P$  represents plasma gravity and  $G_B$  the whole blood gravity. Equation 3 is derived from Equation 2 by substituting, for  $G_P$ , its mean normal value, 1.0264.

1. Gm. protein per 100 c.c. of plasma  $\approx 389.6 (G_P - 1.0079)$
2. Gm. hemoglobin per 100 c.c. of blood  $\approx 33.9 \frac{G_B - G_P}{1.0970 - G_P}$
3. (Approximate hemoglobin from gravity of whole blood alone)  
Gm. hemoglobin per 100 c.c. of blood  $\approx 480 (G_B - 1.0264)$
4. Hematocrit (c.c. cells per 100 c.c. blood)  $\approx \frac{100 (G_B - G_P)}{1.0970 - G_P}$

These calculations can be made graphically by the line charts of Phillips and associates.<sup>1, 2</sup>

TABLE II. COMPARISON OF PLASMA AND BLOOD SPECIFIC GRAVITIES, AND THE CORRESPONDING PLASMA PROTEIN AND HEMOGLOBIN CONCENTRATIONS, AS DETERMINED WITH THE COPPER SULFATE METHOD BY TWO OBSERVERS ON THE SAME SPECIMENS (BLOOD SAMPLES DRAWN FROM UNSELECTED CASES ON MEDICAL WARDS)

CASE	SPECIFIC GRAVITY OF WHOLE BLOOD		SPECIFIC GRAVITY OF PLASMA		PLASMA PROTEINS (GM. PER 100 C.C. PLASMA)		HEMOGLOBIN (GM. PER 100 C.C. BLOOD)	
	ANALYST C	ANALYST D	ANALYST C	ANALYST D	ANALYST C	ANALYST D	ANALYST C	ANALYST D
1	1.0523	1.0520	1.0265	1.0265	7.23	7.23	12.42	12.25
2	1.0513	1.0510	1.0290	1.0295	8.21	8.40	11.12	10.80
3	1.0565	1.0560	1.0270	1.0265	7.43	7.23	14.28	14.18
4	1.0565	1.0565	1.0305	1.0303	8.79	8.71	13.24	13.25
5	1.0532	1.0530	1.0280	1.0280	7.81	7.81	12.38	12.28
6	1.0530	1.0530	1.0300	1.0305	8.59	8.79	11.64	11.47
7	1.0492	1.0498	1.0232	1.0235	5.96	6.07	11.94	12.14
8	1.0610	1.0600	1.0420	1.0428	13.28	13.59	11.72	10.77
9	1.0543	1.0540	1.0280	1.0277	7.81	7.70	12.92	12.86
10	1.0620	1.0620	1.0280	1.0278	7.81	7.74	16.72	16.76
11	1.0500	1.0497	1.0283	1.0277	7.93	7.70	10.55	10.76
12	1.0587	1.0593	1.0305	1.0308	8.80	8.90	14.37	14.49
13	1.0542	1.0545	1.0260	1.0258	7.04	6.97	13.46	13.66
14	1.0367	1.0365	1.0232	1.0235	5.96	6.07	6.19	6.00
15*	1.0545	1.0543	1.0271	1.0290	7.47	8.20	13.29	12.60
16	1.0651	1.0655	1.0288	1.0285	8.13	8.01	18.05	18.31
17	1.0558	1.0558	1.0268	1.0270	7.36	7.43	14.00	13.95
18	1.0577	1.0575	1.0310	1.0305	9.00	8.80	13.72	13.76
19	1.0582	1.0582	1.0280	1.0280	7.82	7.82	14.84	14.84
20	1.0585	1.0580	1.0290	1.0293	8.21	8.32	14.71	14.36
21	1.0435	1.0439	1.0252	1.0250	6.73	6.66	8.64	8.48
22	1.0475	1.0478	1.0290	1.0290	8.20	8.20	9.22	9.38
23	1.0620	1.0618	1.0301	1.0302	8.63	8.67	16.16	16.04
24	1.0522	1.0516	1.0273	1.0270	7.55	7.43	12.11	11.91
25	1.0565	1.0563	1.0270	1.0268	7.43	7.36	14.28	14.24
26	1.0552	1.0552	1.0272	1.0272	7.50	7.50	13.59	13.59
27	1.0528	1.0530	1.0260	1.0260	7.04	7.04	12.80	12.89
28	1.0495	1.0490	1.0275	1.0275	7.73	7.63	10.73	10.49
29	1.0603	1.0600	1.0281	1.0283	7.84	7.93	15.85	15.64
30	1.0588	1.0590	1.0282	1.0282	7.90	7.90	15.07	15.17
31	1.0520	1.0523	1.0277	1.0280	7.71	7.82	11.88	11.95
32	1.0590	1.0585	1.0280	1.0276	7.82	7.66	15.24	15.19
33	1.0585	1.0588	1.0287	1.0288	8.10	8.13	14.80	14.92
34	1.0421	1.0421	1.0198	1.0197	4.63	4.60	9.75	9.82
35	1.0443	1.0440	1.0270	1.0261	7.43	7.08	8.38	8.56
36	1.0587	1.0585	1.0272	1.0270	7.50	7.43	15.30	15.25
37	1.0582	1.0582	1.0282	1.0282	7.90	7.90	14.78	14.78
38	1.0605	1.0603	1.0275	1.0279	7.62	7.78	16.10	15.90
39	1.0523	1.0525	1.0260	1.0264	7.04	7.20	12.56	12.53
40	1.0505	1.0508	1.0254	1.0255	6.81	6.84	11.88	11.94
41	1.0491	1.0490	1.0230	1.0227	5.88	5.77	11.85	12.00
42	1.0642	1.0642	1.0283	1.0282	7.94	7.90	17.73	17.75
43	1.0568	1.0568	1.0255	1.0258	6.85	6.96	14.84	14.76
44	1.0507	1.0505	1.0301	1.0301	8.63	8.63	10.44	10.33
45	1.0411	1.0414	1.0228	1.0228	5.81	5.81	8.36	8.50
46	1.0377	1.0375	1.0210	1.0208	5.11	5.04	7.45	7.43
47	1.0515	1.0515	1.0255	1.0256	6.85	6.89	12.33	12.33
48	1.0580	1.0583	1.0275	1.0275	7.62	7.62	14.87	15.02
49	1.0584	1.0582	1.0273	1.0270	7.55	7.43	15.13	15.32
50	1.0572	1.0575	1.0249	1.0250	6.61	6.65	15.18	15.31
Mean difference	0.0003		0.0002		0.08		0.12	
Maximal difference	0.0010		0.0009		0.32		0.95	
96 per cent of duplicates are within	0.0006		0.0005		0.27		0.32	

\*Results from Case 15 for plasma gravity, plasma proteins, and hemoglobin are excluded from calculation of mean, maximal, and 96 per cent differences because of obvious error in one of the plasma gravities in this case.

The agreement of results obtained by different analysts was close enough (Table II) to demonstrate that the copper sulfate method provides the required degree of constancy for clinical work.

*Comparison of Results by the Copper Sulfate Method With Results by the Barbour Falling Drop Method.*—The method of Barbour and Hamilton<sup>5</sup> has been in use for a number of years for estimating the specific gravity of plasma, and its accuracy has been satisfactorily demonstrated. In Table III results by this method applied to a number of plasmas are compared with results by the copper sulfate method. The agreement between the two is practically as close as the agreement between duplicates by either method.

TABLE III. COMPARISON OF PLASMA PROTEIN CONCENTRATIONS CALCULATED FROM GRAVITIES DETERMINED BY THE COPPER SULFATE METHOD AND THE BARBOUR FALLING DROP METHOD, RESPECTIVELY

CASE	PLASMA PROTEINS (GM. PER 100 C.C.)*	
	FROM GRAVITY BY $\text{CuSO}_4$ METHOD†	FROM GRAVITY BY BARBOUR METHOD
	a	b
1	7.2	7.1
2	8.3	8.3
4	8.8	8.9
6	8.7	8.5
8	13.4	13.4
18	8.9	8.9
22	8.2	8.2
27	7.0	7.3
28	7.7	7.7
34	4.6	4.9
36	7.5	7.5
38	7.7	7.8
40	6.8	6.7

\*Protein values from gravities by both methods are calculated as  
Gm. protein per 100 c.c. =  $389.6 (G_p - 1.0079)$ .

†Mean of analysis C and D, Table II

*Comparison of Serum Protein Concentrations Calculated From Copper Sulfate Gravity Determination With Proteins Determined by Kjeldahl Analysis.*—The results of this comparison are given in Table IV. The micro-Kjeldahl analyses were done with distillation and titration of the ammonia, the protein

TABLE IV. COMPARISON OF SERUM PROTEIN CONCENTRATIONS CALCULATED FROM SPECIFIC GRAVITIES BY THE COPPER SULFATE METHOD WITH CONCENTRATIONS DETERMINED BY MICRO-KJELDAHL ANALYSIS

CASE (TABLE II)	SERUM PROTEINS			OTHER BLOOD CONSTITUENTS	
	FROM GRAVITY BY $\text{CuSO}_4$ METHOD* (GM. PER 100 C.C.)	FROM KJELDAHL NITROGEN (GM. PER 100 C.C.)	DIFFERENCE, GRAVITY MINUS KJELDAHL (GM. PER 100 C.C.)	PLASMA GLOBULINS (GM. PER 100 C.C.)	BLOOD UREA NITROGEN (MG. PER 100 C.C.)
1	7.0	7.1	-0.1	4.9	5
2	8.1	7.4	+0.7	3.6	7
4	8.6	8.1	+0.5	—	36
6	8.5	8.0	+0.5	3.7	9
8	13.2	12.8	+0.4	9.2	5
18	8.7	9.0	-0.3	4.4	13
22	8.0	7.6	+0.4	3.5	6
27	6.8	7.0	-0.2	3.1	11
28	7.4	7.1	+0.3	3.3	10
34	4.5	5.3	-0.8	2.6	14
36	7.3	7.1	+0.2	4.0	6
38	7.5	7.0	+0.5	3.3	11
40	6.7	6.5	+0.2	4.5	5
44	8.4	8.6	-0.2	4.3	11
Mean difference			+0.15		

\*The serum gravity was estimated at 0.0005 less than the mean of the duplicate plasma gravities of each case (Table II), in accordance with the statement of Phillips and co-workers, that the gravity of serum averages 0.0005 less than the gravity of plasma.



nitrogen being estimated as the difference between the total serum nitrogen and the nonprotein nitrogen and multiplied by 6.25 to calculate weight of protein. The nitrogen figures were available on serum, while the gravities were determined on plasma of the same blood specimens. However, the deviation of plasma gravity from serum gravity is so small that correction for the difference can be made with a fair degree of accuracy, as stated by Phillips and co-workers, by subtracting 0.0005 from plasma gravity to obtain serum gravity. From the serum gravities thus obtained the serum proteins were calculated by Equation 1.

In twelve of the fourteen cases, protein concentration estimated by gravity agreed within  $\pm 0.5$  Gm. per 100 c.c., with protein estimated by Kjeldahl; in the other two cases the differences, gravity minus Kjeldahl, were  $+0.7$  and  $-0.8$  Gm. The gravity method appears to be adequate for diagnosis of hyper- and hypo-proteinemia.

*Accuracy of Hemoglobin Values Estimated From Gravities of Whole Blood Alone.*—For accurate estimation of hemoglobin from gravity values (Equation 2), it is necessary to use the gravities of both whole blood and plasma, since the hemoglobin calculation is based partly on the difference between these two values. However, as pointed out by Phillips and associates,<sup>2</sup> the effects of plasma gravity variations on the calculated hemoglobin values are usually small in comparison with the effect of whole blood gravity variations, so that in normal and most pathologic bloods approximate hemoglobin calculations can be made from whole blood gravity alone (by Equation 3) with the assumption that the plasma gravity has the mean normal value 1.0264. This procedure has the advantage that it enables one to make a bedside estimate of hemoglobin from finger blood. The results obtainable in clinical material are exemplified by the data in Table V. They show that in forty-seven of fifty cases the hemoglobin estimated from whole blood gravity alone was within 1.5 Gm. of the hemoglobin more exactly calculated from the gravities of both whole blood and plasma. In cases of hyperglobulinemia, however, such as may occur in multiple myeloma, plasma proteins may be so high that a gross error is caused in hemoglobin calculated by Equation 3. An example is Case 8, in which the plasma proteins were 12.8 Gm. per 100 c.c. (Table IV) instead of the assumed normal 7.2 Gm. The extra 5.6 Gm. of plasma protein caused a plus error of 4.7 Gm. in the hemoglobin estimated from whole blood gravity alone. Except in such gross hyperproteinemia, which appears to occur in about 1 per cent of patients on medical wards,<sup>6</sup> it appears that the whole blood gravity by itself may be used for approximate hemoglobin estimations, reliable within  $\pm 2$  Gm. per 100 c.c.

*Comparison of Hematocrit Values Calculated From Copper Sulfate Gravities With Hematocrit Values Determined by Centrifuge.*—The results of this comparison are given in Table VI. Phillips and co-workers specify for hematocrit determinations centrifugation for sixty minutes at 3,000 r.p.m., with a radius of 18 cm. from the axis of the centrifuge to the center of the hematocrit tube. In the present analyses centrifugation was for the specified sixty minutes at 3,000 r.p.m., but the radius was shorter than 18 cm., so that the centrifugal force was less, and the cell columns obtained were somewhat longer. However, the ratio of column readings with the short-radius centrifuge to readings obtained with the standard 18 cm. radius was found to be constant and was applied to correct the observed readings. The centrifuge hematocrit values given in Table VI are those estimated for the standard conditions with a centrifuge of 18 cm. radius.

TABLE V. COMPARISON OF (a) HEMOGLOBIN CALCULATED FROM OBSERVED GRAVITIES OF WHOLE BLOOD AND PLASMA WITH (b) HEMOGLOBIN CALCULATED FROM OBSERVED GRAVITY OF WHOLE BLOOD ONLY, PLASMA GRAVITY BEING ASSUMED TO BE 1.0264 (GRAVITIES USED ARE MEANS OF C AND D, TABLE II; HEMOGLOBIN CALCULATED AS GRAMS PER 100 C.C. OF BLOOD)

CASE	HEMOGLOBIN CALCULATED		
	FROM $G_R$ AND $G_P$ BY EQUATION 2	FROM $G_R$ ALONE BY EQUATION 3	DIFFERENCE
	a	b	b-a
1	12.34	12.49	+ 0.15
2	10.96	12.00	+ 1.04
3	14.23	14.47	+ 0.24
4	13.25	14.57	+ 1.32
5	12.33	12.92	+ 0.59
6	11.56	12.88	+ 1.32
7	12.04	11.18	- 0.86
8	11.75	16.50	+ 4.75
9	12.89	13.41	+ 0.52
10	16.74	17.23	+ 0.49
11	10.66	11.37	+ 0.71
12	14.43	15.77	+ 1.34
13	13.56	13.55	- 0.01
14	6.10	4.94	- 1.16
15	12.95	13.55	+ 0.60
16	18.18	18.83	+ 0.65
17	13.98	14.23	+ 0.25
18	13.74	15.10	+ 1.36
19	14.84	15.39	+ 0.55
20	14.54	15.44	+ 0.90
21	8.56	8.16	- 0.40
22	9.30	10.31	+ 1.01
23	16.10	17.18	+ 1.08
24	12.01	12.38	+ 0.37
25	14.26	14.52	+ 0.26
26	13.59	13.94	+ 0.35
27	12.85	12.82	- 0.03
28	10.61	11.08	+ 0.47
29	15.75	16.31	+ 0.61
30	15.12	15.73	+ 0.61
31	11.92	12.48	+ 0.56
32	15.22	15.68	+ 0.46
33	14.86	15.63	+ 0.77
34	9.79	7.60	- 2.19
35	8.47	8.62	+ 0.15
36	15.28	15.58	+ 0.30
37	14.78	15.38	+ 0.60
38	16.00	16.46	+ 0.46
39	12.55	12.58	+ 0.03
40	11.91	11.76	- 0.15
41	11.93	10.98	- 0.95
42	17.74	18.30	+ 0.56
43	14.80	14.71	- 0.09
44	10.38	11.72	+ 1.34
45	8.43	7.22	- 1.21
46	7.44	5.42	- 2.02
47	12.33	12.15	- 0.18
48	14.93	15.38	+ 0.45
49	15.23	15.44	+ 0.21
50	15.25	15.00	- 0.25

Summary.—Deviations of hemoglobin estimated approximately by gravity of whole blood alone, from hemoglobin calculated more exactly by gravities of both whole blood and plasma, were grouped as follows:

Deviations under 1 Gm., 37 cases (74 per cent)  
 Deviations under 1.5 Gm., 47 cases (94 per cent)  
 Deviations under 2.2 Gm., 49 cases (98 per cent)  
 Case 8 had gross hyperproteinemia, 12.8 Gm.  
 protein per 100 c.c. plasma.

The agreement between gravity-estimated and centrifuged hematocrits in our pathologic bloods is less close than in the normal bloods reported by Phillips and associates; the standard deviation between the two sets of hematocrit values in our patients was  $\pm 2.6$  c.c. of cells per 100 c.c. of blood, compared with  $\pm 1.2$  in the normal bloods reported by Phillips and co-workers. The latter authors

point out that the gravity method is theoretically less accurate for hematocrits than for hemoglobin and that abnormality in erythrocyte specific gravity increases the error of gravity-estimated hematocrit values. Ashworth and Adams found the mean deviation of gravity-calculated hematocrits from centrifuge hematocrits was three times as great in a series of pathologic bloods as in normal bloods.

In the series of fifty-three cases listed in Table VI, fifty-one show gravity-calculated hematocrit values, in cells per 100 c.c. of blood, that are within  $\pm 4.4$

TABLE VI. COMPARISON OF HEMATOCRITS ESTIMATED BY THE COPPER SULFATE METHOD WITH HEMATOCRITS DETERMINED BY CENTRIFUGATION (FIFTY-THREE CASES)

CASE	CUSO <sub>4</sub> HEMATOCRIT a	CENTRIFUGE HEMATOCRIT b	DIFFERENCE a-b
A	31.0	29.6	+ 0.4
B	34.9	34.1	+ 0.8
C	42.5	39.4	+ 3.1
D	47.2	42.8	+ 4.4
E	45.5	42.8	+ 2.7
F	12.0	10.0	+ 2.0
G	28.0	25.4	+ 2.6
H	30.9	33.7	- 2.8
I	36.6	38.3	- 1.7
J	40.3	39.4	+ 0.9
K	21.2	22.8	- 1.6
L	38.0	39.2	- 1.2
M	36.1	33.3	+ 2.8
1	36.4	38.2	- 1.8
2	32.4	35.8	- 3.4
3	42.0	41.6	+ 0.4
4	39.1	39.9	- 0.8
5	36.4	37.8	- 1.4
6	34.1	35.1	- 1.0
7	35.5	36.6	- 1.1
8	34.7	31.4	+ 3.3
9	38.0	38.6	- 0.6
10	49.4	47.6	+ 1.8
11	31.5	33.2	- 1.7
12	42.6	45.7	- 3.1
13	40.0	39.5	+ 0.5
14	18.0	20.7	- 2.7
15	38.2	39.2	- 1.0
16	53.6	49.8	+ 3.8
17	41.3	40.6	+ 0.7
18	40.5	42.1	- 1.6
19	43.7	41.7	+ 2.0
20	43.0	43.6	- 0.6
21	25.5	25.1	+ 0.4
22	27.4	30.2	- 2.8
23	47.5	47.0	+ 0.5
24	35.4	36.1	- 0.7
25	42.1	41.6	+ 0.5
26	40.1	39.1	+ 1.0
27	38.2	40.5	- 2.3
28	31.3	30.0	+ 1.3
29	46.5	44.6	+ 1.9
30	44.6	35.2	+ 9.4
31	35.1	43.3	- 8.2
33	43.9	44.3	- 0.4
34	28.9	30.2	- 1.3
35	25.0	25.1	- 0.1
36	45.1	46.7	- 1.6
38	47.2	43.4	+ 3.8
39	37.0	39.6	- 2.6
40	35.3	35.3	0.0
41	35.2	36.4	- 1.2
42	52.3	50.6	+ 1.7
Mean difference			+ 0.06
Standard deviation from mean difference			$\pm 2.6$
Deviations under $\pm 4.0$ c.c. in fifty of fifty-three cases.			

c.c. of the values determined by centrifuge and could be considered usable for ordinary clinical purposes. In the other two cases, however, the deviations were too great; namely, +9 and -8 c.c., respectively.

If the small proportion (4 per cent in our series) of patients with abnormalities like these two are excluded by preliminary comparisons of gravity and cen-

TABLE VII. COMPARISONS OF RED BLOOD CELL COUNTS AS DETERMINED BY DIRECT CHAMBER COUNT AND AS ESTIMATED FROM THE HEMOGLOBIN DETERMINED BY THE COPPER SULFATE METHOD; DUPLICATE DETERMINATIONS DONE BY DIFFERENT OBSERVERS (FIGURES FOR COUNTS REPRESENT MILLIONS OF CELLS PER CUBIC MILLIMETER)

CASE	R.B.C. CHAMBER COUNT			R.B.C. CALCULATED FROM CuSO <sub>4</sub> GRAVITIES			DEVIATION OF AVER- AGE CuSO <sub>4</sub> R.B.C. FROM AVERAGE CHAMBER COUNT R.B.C.
	OBSERVER A OR C	OBSERVER B OR D	DIFFER- ENCE	OBSERVER A OR C	OBSERVER B OR D	DIFFER- ENCE	
Normocytic Blood, (Twenty Specimens)							
A	4.9	4.9	0.0	5.4	5.2	0.2	+0.3
3	4.5	4.2	0.3	4.6	4.6	0.0	+0.25
4	4.0	3.9	0.1	4.3	4.3	0.0	+0.45
5	4.5	4.8	0.3	4.0	4.0	0.0	-0.65
7	3.7	3.4	0.3	3.9	3.9	0.0	+0.25
9	4.2	4.3	0.1	4.2	4.2	0.0	-0.05
10	-	5.8	-	5.4	5.4	0.0	-0.4
11	3.3	4.0	0.7	3.4	3.5	0.1	-0.2
13	4.9	4.9	0.0	4.4	4.4	0.0	-0.5
16	5.8	5.8	0.0	5.9	5.9	0.0	+0.1
17	4.9	4.4	0.5	4.5	4.5	0.0	-0.15
19	5.0	4.5	0.5	4.8	4.8	0.0	+0.05
20	3.9	4.2	0.3	4.8	4.7	0.1	+0.7
25	4.7	4.6	0.1	4.6	4.6	0.0	-0.05
27	4.6	4.2	0.4	4.2	4.2	0.0	+0.2
29	4.7	5.1	0.4	5.1	5.1	0.0	+0.2
33	4.8	4.6	0.2	4.8	4.9	0.1	+0.15
34	3.5	3.6	0.1	3.2	3.2	0.0	-0.25
44	3.4	3.7	0.3	3.4	3.4	0.0	-0.15
46	2.2	2.5	0.3	2.4	2.4	0.0	+0.05
Average difference			0.3			0.02	0.0
Spread			0.0 to			0.0 to	+0.7 to
			0.7			0.2	-0.65
Hyperchromic Blood, (Fourteen Specimens)							
C	4.5	4.6	0.1	5.1	5.1	0.0	+0.55
D	4.5	4.9	0.4	5.0	5.0	0.0	+0.3
E	3.6	3.9	0.3	4.5	4.4	0.1	+0.7
F	3.9	3.4	0.5	3.9	3.9	0.0	+0.25
H	4.7	5.1	0.4	5.6	5.5	0.1	+0.65
I	3.0	3.1	0.1	3.5	3.6	0.1	+0.50
1	3.0	3.0	0.0	4.0	4.0	0.0	+1.0
6	3.3	3.1	0.2	3.8	3.7	0.1	+0.55
12	3.6	3.9	0.3	4.7	4.7	0.0	+0.95
14	1.5	1.4	0.1	2.0	1.9	0.1	+0.5
15	3.6	-	-	4.3	4.1	0.2	+0.6
21	2.7	-	-	2.8	2.8	0.0	+0.1
36	5.0	4.3	0.7	5.0	5.0	0.0	+0.35
40	3.2	3.7	0.5	3.9	3.9	0.0	+0.45
Average difference			0.3			0.04	+0.54
Spread			0.0 to			0.0 to	+0.1 to
			0.7			0.2	+1.0
Hypochromic Blood, (Nine Specimens)							
8	4.7	4.1	0.6	3.8	3.5	0.3	-0.65
18	5.5	5.3	0.2	4.5	4.5	0.0	-0.9
22	3.9	4.0	0.1	3.0	3.0	0.0	-0.95
23	4.9	4.9	0.0	5.2	5.2	0.0	+0.3
24	-	4.1	-	3.9	3.9	0.0	-0.2
28	3.2	3.5	0.3	3.5	3.4	0.1	-0.1
35	3.5	3.6	0.1	2.7	2.8	0.1	-0.8
38	-	5.0	-	4.1	4.1	0.0	-0.9
Average difference			0.2			0.05	-0.55
Spread			0.0 to			0.0 to	+0.3 to
			0.6			0.3	-0.95

trifuge hematocrit values, it appears that in patients not thus excluded the gravity method can be used in following the subsequent clinical course of hematocrit changes.

*Cell Counts Estimated From Gravities.*—In bloods with a normal ratio of hemoglobin to cell count, the count can be calculated by Equation 5, in which the constant, 0.325, has been computed from data in the present paper.

$$5. \text{ Million R.B.C. per mm.}^3 = \text{Hb} \times 0.325$$

where Hb. represents grams of hemoglobin per 100 c.c. of blood, estimated from copper sulfate determinations by Equation 2.

In Table VII is presented a comparison of values calculated by this equation with chamber counts on the same patients. Duplicate measurements were made by different observers. All bloods in the series with a color index between 0.9 and 1.1 were considered normocytic; no blood films were made.

Since in Equation 5 the assumption is made that the cells have mean normal values for both size and hemoglobin concentration, one would expect that hyperchromic bloods would give red cell counts which are too high, when calculated from Hb., and hypochromic bloods the converse. That this is true is seen from Table VII. Abnormality in the color index eliminates the usefulness of cell counts estimated from gravities.

The average difference between duplicate copper sulfate red cell counts is less than that between duplicate chamber counts. Results are therefore more exactly reproducible by the gravity method than by the chamber count.

Phillips and associates do not suggest estimating cell counts from gravities, but our results indicate that when a patient shows a normal color index (ratio of hemoglobin to cell count), the rapid gravity method can be used to estimate changes in cell count during treatment.

#### CONCLUSIONS

The copper sulfate method for specific gravities of whole blood and plasma, tested in the medical service of a general hospital, proved to be simple and accurate and to provide a quick procedure for estimation of hemoglobin, plasma protein, and, except in specific types of cases, hematocrit and cell count.

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## THE THIAMINE REQUIREMENT IN ALLOXAN DIABETES

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PERIPHERAL neuritis is one of the frequent complications of diabetes. The factors underlying the development of this condition are as yet undetermined, but the possibility that thiamine may be involved has repeatedly been raised.<sup>1-3</sup> The known function of thiamine in the metabolism of carbohydrate,<sup>4</sup> together with its role in the development of certain forms of neuritis, has led some authors to believe that the requirement of this vitamin may be raised in diabetes and that the neuropathies are due to thiamine deficiency.<sup>5</sup> On the other hand, the diminished oxidation of carbohydrate which presumably takes place in diabetes would be expected to decrease the body's requirement. Alloxan diabetes offers a ready tool for the investigation of this problem. The following study was undertaken to determine if rats made diabetic by the injection of alloxan have a greater tendency to develop thiamine deficiency than normal controls and whether their requirement for thiamine is appreciably different from normal animals. A somewhat similar study has been reported by Styron and co-workers<sup>6</sup> using depancreatized rats, but the diabetes in their animals was very mild compared to the alloxan diabetes used in the following experiments, and no attempt was made by us to determine the effect of diabetes on the thiamine requirement.

### EXPERIMENTAL

Adult male rats weighing between 250 and 350 grams were made diabetic by the subcutaneous injection of alloxan. A dose of 200 mg. per kilogram of body weight was given to the first ten animals, but nine of these died on the second day. Subsequently, the initial injections were reduced to one-half this dose. In animals resistant to this treatment, repeated daily injections gradually increasing up to 150 mg. per kilogram of body weight were made after a lapse of two or three days and were continued until the urine showed a consistent 4 plus reaction to Benedict's solution. Of thirty-nine rats treated, there were only eight fatalities by this technique, and none proved ultimately resistant though in one rat nineteen injections were necessary. There were no relapses from the diabetic to the normal state in this series. Following the injections of alloxan, all the animals suffered an immediate sharp fall in weight but over the course of the next week tended to maintain themselves at a new and, in all but one rat, lower level.

As soon as the diabetes in these animals was definitely established or shortly thereafter, they were divided into two groups. Group A consisted of seven rats which were placed on a thiamine-deficient diet composed of 74 per cent sucrose, 18 per cent casein, 4 per cent fat, and 4 per cent salt mixture.<sup>7</sup> Water-soluble vitamins were added to supply 4 mg. pyridoxine hydrochloride, 4 mg. riboflavin, 30 mg. nicotinic acid, 15 mg. calcium pantothenate, and 2.0 Gm. choline chloride per kilogram of ration.\* One drop of haliver oil was given

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\*The synthetic vitamins were generously supplied by Merck & Co., Inc., Rahway, N. J.

twice weekly to supply vitamins A and D. Group B consisted of four diabetic rats which were placed on the same diet plus 1 mg. of thiamine per 100 Gm. of ration. A control group, Group C, consisting of seven normal rats, was also given the thiamine-deficient diet. In the diabetic animals, frequent tests were performed with Benedict's solution to determine urinary sugar, and daily water consumption was measured. Weight changes were recorded daily.

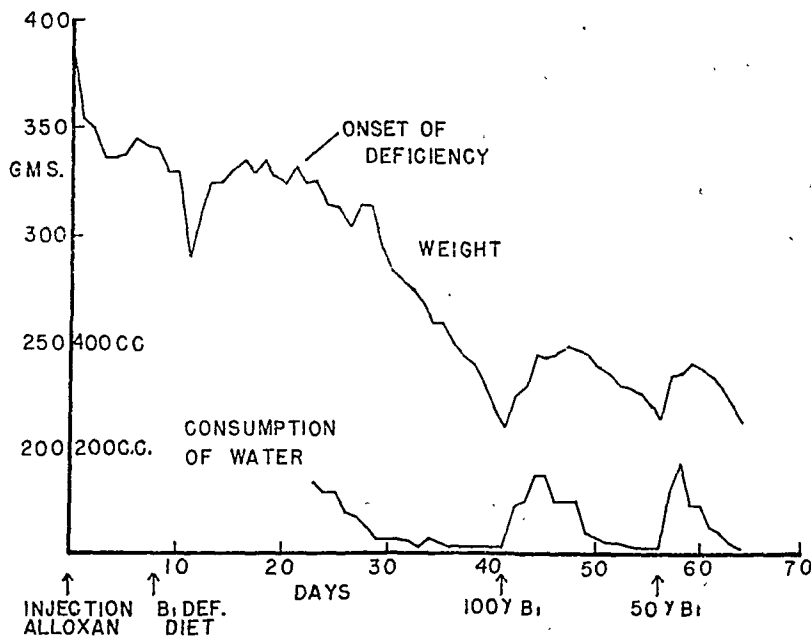


Fig. 1.—Thiamine deficiency in a typical diabetic animal and the response to therapy.

The time required to develop thiamine deficiency was taken as the time after the animals were given the deficient diet until weight loss became consistent. After the animals had lost from one-quarter to one-third of their body weight, the relative thiamine requirement of the diabetic animals versus that of the controls was determined by comparing the gain in weight following intraperitoneal injection of thiamine chloride. After a response to thiamine was obtained and the weight of the animals had fallen to the original level, another injection was given. Thus several responses, usually at different levels of thiamine, were obtained with each rat. In Fig. 1 is shown a typical example of the course followed by a diabetic animal.

#### RESULTS

The rats in Group B which received thiamine appear in good health after a period of four months although they are severely diabetic. They have demonstrated a marked increase in the consumption of both food and water, eating an average of 22 Gm. and drinking an average of 150 c.c. of water per day. Their weights may vary from 3 to 5 grams from day to day, and over a week up to 10 grams, but there has been no significant change following the initial period of stabilization. Their maintenance of weight indicates that marked and continued changes in the animals in Group A can be taken as an index of response to the administration of thiamine.

In Group A, diabetic animals on the thiamine-deficient diet, one rat died after two days. The others were continued on the ration, and after an average

of 16.1 days, developed listlessness and almost a complete loss of appetite and suffered a marked and continuous fall in weight. Water consumption gradually declined to from 2 to 5 c.c. per day, and urinary sugar dropped from a 4 plus to a 3 plus reaction. The normal control animals in Group C, receiving the same ration, developed similar symptoms and weight changes after an average of 14.1 days. After the death of one control, no further attempt was made to await the development of neuritis, but following the loss of not less than 80 grams of weight subsequent to the onset of signs of deficiency, both diabetic animals and controls were injected intraperitoneally with doses of 50, 100, or 200  $\mu$ g of thiamine chloride. There followed an immediate alleviation of symptoms, a gain in weight, and in the diabetic animals a striking increase in the consumption of water and excretion of sugar. The maximum weight gain was found to be the most reliable index of response and the average results obtained at the different levels of thiamine administered are shown in Table I.

TABLE I. AVERAGE RESPONSE TO THIAMINE

THIAMINE DOSE ( $\mu$ G)	KIND OF ANIMALS	NUMBER OF TESTS	AVERAGE MAXIMUM GAIN (GM.)	PER CENT OF MEAN VALUE
50	Diabetic	6	27.8	108
	Control	5	24.0	93
100	Diabetic	7	39.4	116
	Control	4	28.5	84
200	Diabetic	3	46.0	116
	Control	2	33.0	84

The greater response of the diabetic animals at all levels of thiamine is apparently related to the composition of the material metabolized. During a period of four days, the food intake of the diabetic animals in Group B was measured. Sugar excretion<sup>8</sup> and urinary and fecal nitrogen were also measured quantitatively. Since these animals had maintained their weight over a prolonged period on this diet, the amount and composition of food actually metabolized was calculated as the sum of the fat eaten, the protein equivalent to the urinary nitrogen, and the carbohydrate eaten less the sugar excreted in the urine. The caloric requirement thus determined was approximately 40 calories per day, almost exactly the same as has been previously found by food consumption measurements on normal rats of the same weight.\* However, to compensate for the continuous excretion of sugar, the diabetic animals ate approximately twice as much food as normal rats. Thus the composition of the diet actually metabolized was approximately 8 per cent fat and 36 per cent protein, compared to 4 per cent fat and 18 per cent protein in the controls.

## DISCUSSION

The data from this experiment show that the tendency of the diabetic animals to develop thiamine deficiency was approximately the same or perhaps slightly less than the controls. That the thiamine requirement is actually less is convincingly shown by the average responses of the two groups to different levels of thiamine administration. At each level the response of the diabetic animals was greater than that of the controls. The data were analyzed statistically in one group by computing each response as per cent of a mean value midway between the two curves shown in Fig. 2. By the "t" test<sup>9</sup> the differences are highly significant (less than 1 in 500 that differences are due to chance alone).

\*Unpublished data.



The somewhat greater average weight of the controls at the time thiamine was given does not appear to have been an important variable since no correlation between weight at the time of administration and subsequent response was found.

The lower thiamine requirement of the diabetic animals is logically supported by the data upon the composition of the diet metabolized. Both fat and protein have been shown to have a thiamine-sparing action.<sup>10, 11</sup> Wainio<sup>11</sup> has estimated that at a maximum, casein is 64 per cent glycogenic, and he employs a figure of 10 per cent for fat. Using these figures, it may be estimated that the thiamine requirement of the diabetic animals is approximately 12 per cent. less than that of the control animals.

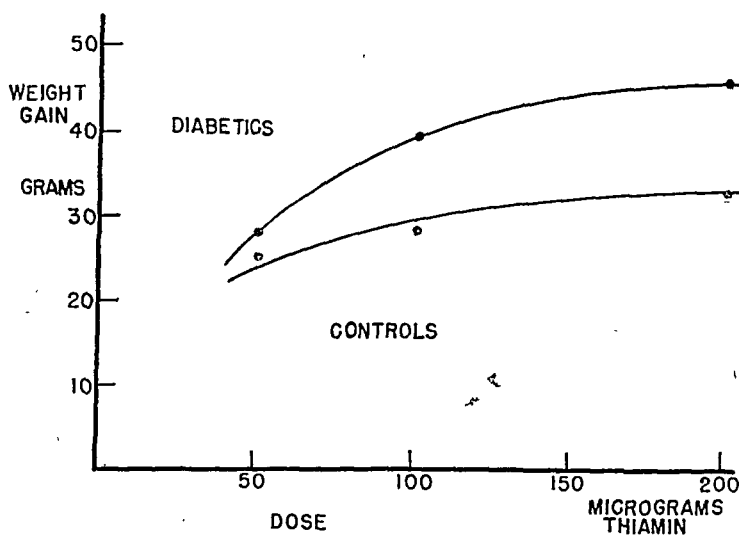


Fig. 2.—A comparison of the response obtained in diabetic versus control animals to graded levels of thiamine.

The findings in this experiment are of additional interest in view of the possibility that marked or prolonged diuresis may tend to "wash out" the body's stores of thiamine. Both prior to the onset of symptoms of deficiency and immediately following the administration of thiamine, the diabetic animals were drinking and excreting the equivalent in a 70 kilogram man of 30 liters of fluid per day. Furthermore, the injection of a single large dose such as was done here would particularly facilitate loss by this means. In view of the failure of these animals to develop deficiency before the controls and their normal response to thiamine administration, it is reasonable to conclude that either an appreciable amount of thiamine was not washed away or that their requirement is considerably less than indicated previously.

Caution must be used, however, in interpreting such experimental findings in terms of human patients. Alloxan diabetes obviously differs from that found in human beings, particularly with regard to capacity to survive without protection by insulin. Furthermore, Rudy and Epstein<sup>5</sup> have reported partial or complete relief of neuritis in 67 of 100 diabetic patients by the administration of supplementary thiamine. However, the exact basis for this therapeutic response is not clear. The role that diet may play in the pathogenesis of this condition is in dispute. Jordan,<sup>12</sup> reviewing 226 cases at Joslin's clinic, concluded that diet was not a factor, while Rudy and Epstein believe that it cannot be ruled out in 73 per cent of their patients. The latter authors have not, however, indicated the method of dietary analysis by which they reach this conclusion. In addition, the possibility remains that there may be a disturbance in thiamine

utilization in human beings with diabetes, although there is no evidence available to support such a hypothesis at this time.

### CONCLUSION

This study presents evidence that rats made diabetic by injection with alloxan show no increased tendency to develop signs of thiamine deficiency, that the action of thiamine is not perceptibly impaired, and that the diabetic animals' requirement of this vitamin is less than that of normal controls. It does not support the theory that diabetic neuritis is due to thiamine deficiency.

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## BACTERIOSTATIC PROPERTIES OF SULFANILAMIDE AND SOME OF ITS DERIVATIVES

### III. THE CARBOXY-SULFATHIAZOLES AS CHEMOTHERAPEUTIC AGENTS ACTIVE IN THE GASTROINTESTINAL TRACT

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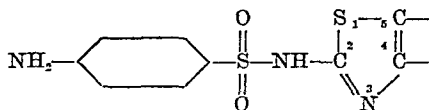
SUCCINYLSULFATHIAZOLE (Sulfasuxidine) was introduced as an intestinal antiseptic by Poth and Knotts in 1941.<sup>1</sup> In 1943, Poth and Ross<sup>2, 3</sup> published the results of a continued study of these dibasic acylated sulfonamides and presented data on phthalylsulfathiazole (Sulfathalidine); this drug was shown to possess approximately twice the bacteriostatic activity of sulfasuxidine when the antibacterial activity was measured against the coliform organisms in the gastrointestinal tract of man and dog. Sulfasuxidine has had extensive

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Aided by a research grant from Sharp and Dohme, Inc., Philadelphia, Pa.

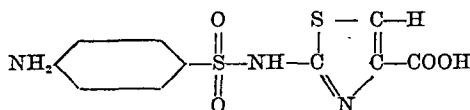
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clinical trial<sup>4,23</sup> and has become well established as a useful drug, being particularly valuable in the treatment of bacillary dysentery and in the preoperative preparation of the bowel. While sulfathalidine has had a less extensive clinical use,<sup>4, 22, 24, 25</sup> it has been shown to be especially valuable in controlling nonspecific diarrheas, chronic ulcerative colitis,<sup>26</sup> and regional enteritis and in the preoperative preparation and postoperative treatment of the bowel. Both of these compounds are finding extensive use in the treatment of chronic urinary tract infections due to the coliform organisms.<sup>24, 27</sup> These two compounds are poorly absorbed from the gastrointestinal tract; this properly would not be anticipated since the molecules contain a free carboxyl group and readily form ionizable salts. The presence of a free carboxyl group has, however, led to the study of another series of sulfonamides, the carboxysulfathiazoles, where the substitutions were on the 4 and 5 carbon atoms of the thiazole rest.



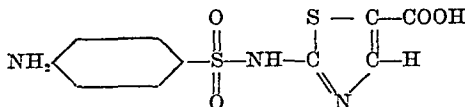
The carboxysulfathiazoles studied were\* (Table I):

1. Sulfa-4, carboxythiazole



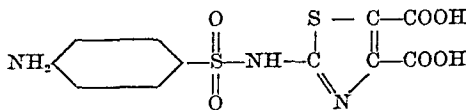
(Mol. wt., 299.3; M. P., 240-241° C. with decomposition; relatively stable chemically)

2. Sulfa-5, carboxythiazole



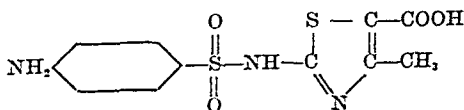
(Mol. wt., 299.3; M. P., 210-215° C. with decomposition; relatively unstable chemically and yields sulfathiazole by splitting out CO<sub>2</sub>)

3. Sulfa-4,5, dicarboxythiazole



(Mol. wt., 343.2; M. P., 240° C. with decomposition; will not form acid salts and will decompose to yield sulfa-4, carboxythiazole)

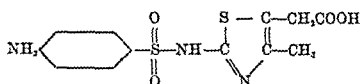
4. Sulfa-4, methyl, 5, carboxythiazole



(Mol. wt., 313.3; M. P., 190-200° C. with decomposition; relatively unstable chemically and will yield sulfa-4, methylthiazole)

\*These compounds were supplied by Sharp and Dohme Inc., Philadelphia, Pa. The authors are indebted to Dr. J. M. Sprague for the chemical data and physical constants presented.

## 5. Sulfa-4, methyl, 5, carboxymethylthiazole



(Mol. wt., 327.3; relatively stable chemically)

These compounds were studied in a manner similar to that of the acylated sulfonamides previously reported.<sup>1, 22</sup> The antibacterial activities of these chemicals, as indicated by the alteration of the coliform organisms in the gastrointestinal tract, were determined by administering them to dogs at four-hour intervals throughout the day and night. The animals were placed on a sub-maintenance diet of boiled horse meat. The drug was mixed with this food, which was made into balls, and fed automatically at four-hour intervals by a device described by Poth.<sup>23</sup> Since the animals always were hungry, they ate their food as soon as it became available so that the drug was ingested regularly.

Stool specimens for bacteriologic study were taken directly from the rectum. The number of coliform organisms was determined by plate counts on desoxycholate media containing 5 mg. per cent of p-aminobenzoic acid.

Blood and urine were analyzed for sulfonamide by modifications of the usual colorimetric procedure. The urine was obtained directly from the bladder by catheter.

The results of these studies are summarized in Table I and Figs. 1, 2, 3.

TABLE I. THE LOCAL ANTIBACTERIAL ACTIVITY OF THE CARBOXYTHIAZOLES IN THE GASTRO-INTESTINAL TRACT OF THE DOG, WITH THE DOSAGE, MAXIMUM CONCENTRATIONS OF THE DRUGS IN THE BLOOD AND URINE, AND THE RELATIVE TOXICITY OF THE SEVERAL COMPOUNDS

DRUG	DAILY DOSAGE* (GM. PER KG.)	MAXIMUM CONCENTRATION OF DRUG IN BLOOD (MG. %)	MAXIMUM CONCENTRATION OF DRUG IN URINE (MG. %)	RELATIVE ANTIBACTERIAL ACTIVITY	RELATIVE TOXICITY
Sulfa-4-carboxythiazole†	0.25 1.0	— 6.0	— 1450	++ +++	0 0
Sulfa-5-carboxythiazole†	0.25 1.0	— 6.5	— 2200	++ ++++	0 0
Sulfa-4,5-dicarboxythiazole†	0.25 0.50 1.0	— 6.2† 6.8†	— 335† 390†	+++ ++++ ++++	0 0 0
Sulfa-4-methyl-5-carboxythiazole	0.5 1.0	10.0 18.0	— 1800	++ +++	0 +++
Sulfa-4-methyl-5-carboxymethylthiazole	0.5 1.0	6.2 6.2	552 554	+++ +++	0 0

\*The daily dose was divided into six equal portions and administered at four-hour intervals.

†Two intravenous doses of 1.0 Gm. each given at twenty-four hour intervals. Drug dissolved by addition of equivalent quantities of NaOH. No toxicity demonstrable.

‡These values are inaccurate because an entirely satisfactory analytical procedure could not be devised.

*In Vitro Activity.*—Doctor W. F. Verwey and co-workers observed the *in vitro* antibacterial activity of the carboxysulfathiazoles as follows: A 1:10,000 dilution of a twenty-hour culture of *Escherichia coli* was cultured in a synthetic medium containing varying amounts of the sulfonamide and taking as the end

point that molar concentration which allowed a growth giving a turbidity equal to 50 per cent of that of the control tube when incubated for twenty hours.

Sulfathiazole	$4 \times 10^{-7}$
Sulfanilamide	$1 \times 10^{-4}$
Succinylsulfathiazole	$7 \times 10^{-4}$
Sulfa-5-carboxythiazole	$8 \times 10^{-5}$
Sulfa-4-carboxythiazole	$6 \times 10^{-4}$
Sulfa-4,5-dicarboxythiazole	$3 \times 10^{-3}$
Sulfa-4-methyl-5-carboxymethylthiazole	$1 \times 10^{-3}$

Comparison of the bacteriostatic activity of these compounds in the intestinal tract, as shown in Table I, with the in vitro antibacterial activity, fails, as is so frequently true, to show any correlation.

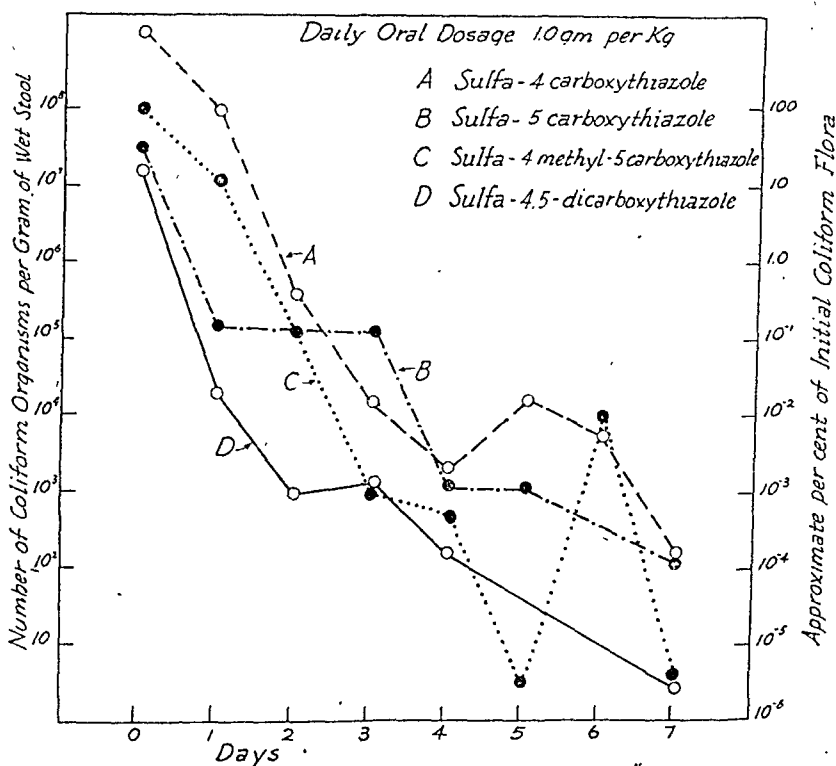


Fig. 1.—The alteration of the coliform flora in the bowel of the dog following the oral administration of carboxysulfathiazoles. At this dosage level, the alterations are identical within the limits of experimental error. The population of these organisms is decreased to 1/100,000,000 of the original.

**Sulfa-4,5-Dicarboxythiazole.**—This compound shows an antibacterial activity against the coliform organisms in the gastrointestinal tract of the dog intermediate between sulfasuxidine and sulfathalidine. Equivalent bacteriostasis occurs following the administration of 1.0 Gm. of sulfasuxidine per kilogram of body weight, 0.5 Gm. sulfa-4,5-dicarboxythiazole, or 0.25 Gm. Sulfathalidine.

Dogs tolerated repeated intravenous doses of 1.0 Gm. per kilogram of sulfa-4,5-dicarboxythiazole without evidence of acute or chronic toxic manifestations.

The intraperitoneal administration of the sodium salt of sulfa-4,5-dicarboxythiazole to mice gives the mortality figures shown in Table II. The lethal dose, 50, is between 1.0 and 1.30 Gm. per kilogram of body weight.

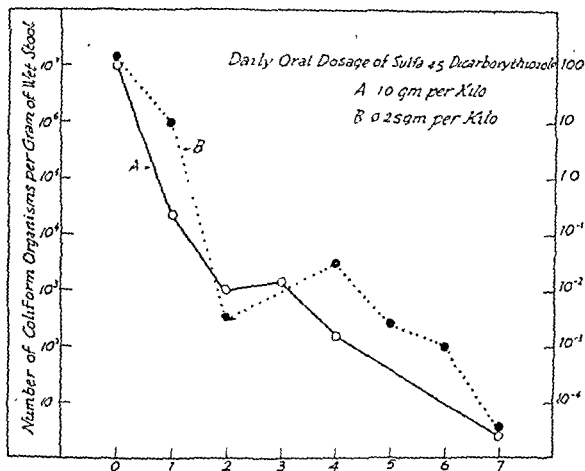


Fig. 2.—Demonstrating that a daily oral dosage of 0.25 Gm. of sulfa-4,5-dicarboxythiazole per kilogram of body weight divided into six portions and given at four-hour intervals to the dog is equally as effective as 1.0 Gm. of the same drug similarly administered.

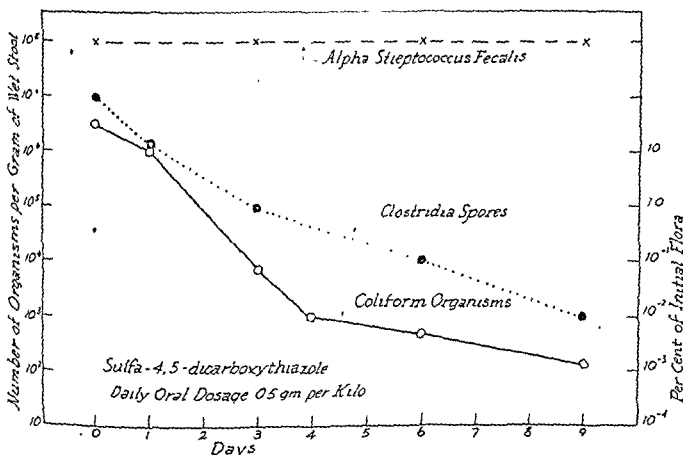


Fig. 3.—Showing the corresponding effect on anaerobic spores and coliform organisms upon the oral administration of sulfa-4,5-dicarboxythiazole to dogs in divided four-hour doses. This drug has no antibacterial action on the alpha *Streptococcus fecalis*.

TABLE II. THE INTRAPERITONEAL ADMINISTRATION OF THE SODIUM SALT OF SULFA-4,5-DICARBOXYTHIAZOLE TO WHITE MICE WEIGHING BETWEEN 29 AND 33 GRAMS

NUMBER OF MICE	DOSAOE (GM. PER KG.)	NUMBER OF MICE ALIVE AFTER FORTY-EIGHT HOURS	MORTALITY (PER CENT)
10	0.67	0	0
10	0.99	1	10
10	1.33	9	90
10	2.66	10	100

## DISCUSSION

The carboxythiazoles contain free carboxyl groups resulting in compounds which are relatively strong acids and which readily form highly soluble salts. In this series of sulfonamides the primary para amino group is free in contradistinction to the acylation of this bacteriostatically active group in the acylated sulfonamides previously studied and represented by succinyl- and phthalylsulfathiazole.

While all of these carboxythiazoles have considerable antibacterial activity in the gastrointestinal tract, sulfa-5-carboxythiazole and sulfa-4,5-dicarboxythiazole possess the greater activity. All of these compounds maintain a moderately high concentration of the drugs in the blood. Comparison of the concentration of the drugs in the urine would indicate that sulfa-5-carboxythiazole is absorbed from the bowel more readily than is sulfa-4,5-dicarboxythiazole (see Table I).

Sulfa-4-methyl-5-carboxythiazole is the only member of this series that has been demonstrated to be relatively toxic. This toxicity is explainable on the basis that the compound readily splits out carbon dioxide to yield the corresponding 4-methylsulfathiazole which is toxic.

The nontoxic members of this series of sulfonamides, which significantly alter the bacterial flora of the gastrointestinal tract as indicated by the reduction of the coliform organisms, may prove to be useful therapeutic agents where it is desired to produce a general systemic effect as well as to alter the intestinal flora and maintain a relatively high concentration of the salt of a sulfonamide in the urinary tract.

## SUMMARY

A series of carboxysulfathiazoles is studied and shown to possess relatively low toxicity and high antibacterial activity in the gastrointestinal tract of the dog. Sulfa-4-carboxythiazole, sulfa-5-carboxythiazole, sulfa-4,5-carboxythiazole, and sulfa-4-methyl-5-carboxymethylthiazole may possess desirable therapeutic properties.

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## MEASUREMENT OF PULMONARY ARTERIAL PRESSURE IN DOGS

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**S**TUDIES of the pressures developed in the pulmonary circulation have been rendered difficult because of the relative inaccessibility of the system. Direct measurements of pulmonary arterial pressures in experimental animals have been carried out,<sup>1, 2</sup> and in one report direct measurement of the pulmonary arterial tension was accomplished in man.<sup>3</sup> More recently, Johnson and co-workers<sup>4</sup> and Katz and Steinitz<sup>5</sup> have studied pulmonary arterial tensions (using special manometers) in trained dogs with closed thoraces. Their method appears to provide a reasonably accurate technique for recording such pressures, although requiring previous surgical preparation.

Our interest in alterations of pulmonary arterial tension in pulmonary embolism and pulmonary edema has led us to devise a method of registration of the tension which is simple and is performed without surgical manipulation of the thorax. The method is essentially a modification of that of Forssmann,<sup>6</sup> later utilized by others<sup>7</sup> for the recording of right intraventricular pressures.

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in the axial stream. Accordingly, the longitudinal pressure curves obtained are smooth in contour and are devoid of abrupt and minute oscillations. The curves are in general conformity to those recorded by Katz and Steinitz<sup>5</sup> and others.<sup>4</sup>

#### DISCUSSION

A question may be raised as to whether the passage of the catheter through the pulmonary valve produces pulmonary insufficiency, at the sacrifice of diastolic pressure head. This possibility was readily investigated. In anesthetized dogs, with the thorax *opened*, an optical manometer was connected to a cannula tied into a small lateral branch of the pulmonary arterial system, and the pressure was recorded. Thereafter, the urethral catheter was introduced into the pulmonary artery by the technique described. The pulmonary arterial tension through the catheter was recorded on the same manometer. With either of these procedures, the mean arterial tensions were the same; the pulse pressure measured through the catheter (longitudinal pressure) was slightly lower than that recorded from a side branch because minute abrupt oscillations were not present. It appears probable that no leak occurs in the valve and that the resilient valve leaflets form an effective seal around the tube.

The manometric system, incorporating the catheter, seemed adequate for this purpose. The sudden application of pressure to the system (100 mm. Hg or more) caused as prompt and complete a deviation of the light beam as was recorded when the same pressure was applied to the manometer through ordinary pressure tubing. Lateral distention appeared to be nil in the catheters.

In numerous trials, verified by autopsy, it was found that, when the catheter tip lies immediately within the cardiac shadow in the pulmonary artery (right or left lateral view), it is in the main trunk of the artery or in the orifice of the left main branch. If the catheter is permitted to proceed farther into one of the lobar branches, it may become impacted in the lumen so that the pressure pulses are not obtained. In addition, it was found that the method is not satisfactory in very small animals, as the catheter will not loop sharply in a small right ventricle and the tip cannot be pushed to the pulmonary orifice; the tube coils upon itself within the heart. As a further precaution, overanesthetization of the animals is to be avoided because of the likelihood of the heart becoming hypodynamic or because respiration may become so altered as to distort the pulmonic pressure readings.

This method lacks the advantage of that of Johnson and co-workers<sup>4</sup> and of Katz and Steinitz<sup>5</sup> in that it requires anesthetization of the dog. In experimentation where anesthesia is desired, the method is advantageous in that it is simple to perform and all previous intrathoracic surgical manipulation is eliminated. It is suggested, further, that the technique might be adapted for the measurement of pulmonic arterial tension in man.

#### SUMMARY

A method is described for the insertion of a soft rubber urethral catheter into the right ventricle and thence into the pulmonary artery of dogs. Pulmonary arterial pressure may be recorded by an optical manometer. The simplicity of the procedure renders it advantageous where such experimentation on the animal is carried out under anesthesia.

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## LIVER FUNCTION STUDIES ON SOLDIERS UNDER PROLONGED ATABRINE ADMINISTRATION

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**A**NIMAL studies have revealed that orally administered atabrine produces liver damage.<sup>1,2</sup> To be sure, the doses employed were massive and in no way to be compared with the daily intake of our soldiers. However, since in this theater of operations, atabrine administration has been in effect for a considerable time the question arose as to whether small amounts of the drug taken over a long period of time might produce a subclinical hepatic damage which could be detected by sensitive liver function studies.

To this end, we took fifty ambulatory Negro soldiers who had been taking 0.1 Gm. of atabrine daily six days a week for about eighteen months. Only those soldiers who never had had clinical malaria, syphilis, scrub typhus, or jaundice were included in the study. The tests employed were icterus index, urine urobilinogen, fibrinogen, serum cholesterol partition, and the oral hippuric acid test. Total serum lipid determinations were performed where the total serum cholesterol values were unusually high. As a check on the fibrinogen results, this determination was also performed on fifty troops who had been taking atabrine only from one to three months.

Several months later, bromsulfalein, cephalin flocculation, and galactose tolerance tests were performed on fifty more soldiers (white and Negro). The latter likewise had been taking atabrine for from twenty to twenty-four months and had no previous history of liver damage. As a control of the sensitivity of the cephalin flocculation test, determinations were performed on twenty-five soldiers who had been taking atabrine for from one to three months. At a still later date, twenty-five additional bromsulfalein tests were performed on soldiers who had been taking atabrine for thirty months.

### METHODS

Plasma fibrinogen was determined colorimetrically.<sup>3-5</sup> Icterus index was determined by the method of Meulengracht.<sup>6</sup> Urine urobilinogen was determined quantitatively by the method of Sparkman.<sup>7</sup> The oral hippuric acid test was

performed according to the method of Quick,<sup>8</sup> as modified by Kraus and Dulkan.<sup>9</sup> Total serum cholesterol was measured colorimetrically,<sup>10</sup> while free cholesterol was determined essentially by the method of Schonheimer and Sperry.<sup>11</sup> The total lipid content of serum was determined gravimetrically.<sup>12, 13</sup> The brom-sulfalein test was performed by injecting 2 mg. of bromsulfalein per kilogram of body weight<sup>14</sup> and by measuring the retention at the end of eighteen minutes.<sup>15</sup> For the cephalin flocculation test, the original method of Hanger<sup>16</sup> was used. The galactose tolerance test was performed by the method of Janney and Isaacson.<sup>17</sup>

#### RESULTS

Icterus index results were all normal, lying between 4 and 8 units. Urine urobilinogen was considerably less than the upper normal value of 8 mg. per cent; the results lay between 0.7 and 3.6 mg. per cent. Serum cholesterol partitions were also normal; the values obtained all were within the normal limits of from 21 to 29 per cent of free cholesterol. Of the fifty oral hippuric acid liver function tests performed, excretions of less than 3 Gm. were obtained in three subjects. Over one-half of the plasma fibrinogen values were less than 0.3 Gm. per cent. However, of fifty control observations, 32 per cent were likewise less than 0.3 Gm. per cent. Therefore, the low plasma fibrinogen cannot be attributed to the effect of atabrine, but to some other unexplained cause. Galactose tolerance studies were all normal. In every instance, 3 Gm. or less of galactose were recovered in the urine within five hours. No retention of brom-sulfalein was observed in any case, not even in those men who had been taking atabrine for thirty months.

At the end of twenty-four hours the cephalin flocculation tests gave a precipitation of 2 plus or greater in 46 per cent of the men, while of the twenty-five control determinations performed, a precipitation of 2 plus or greater was obtained in seven of the subjects. Since almost as high a percentage of positive results was obtained among the controls as among the soldiers studied, it must be concluded that in our hands the cephalin flocculation test was too sensitive and that the positive results obtained are not indicative of hepatic dysfunction. It might be added that the tubes were exposed to light in the laboratory after being set up. This might account for the increased sensitivity.

Consequently, of the hepatic function tests performed, only the hippuric acid test gave results indicative of hepatic dysfunction, and then only in a very few cases. This by itself cannot be considered significant. Therefore, it may be concluded that no subclinical liver damage could be detected among the soldiers that were studied.

Of the total cholesterol results obtained on the fifty Negro soldiers, more than one-third were higher than the usually accepted upper normal limit of 275 mg. per cent. These findings were not expected, and as a check, total serum lipid determinations were performed. In every instance where the cholesterol was high, a slightly elevated serum lipid was obtained. However, the total cholesterol values obtained on the second series of fifty soldiers ran much lower, only one being higher than 275 mg. The only explanation that can be offered for this difference is that the second series of determinations was performed on fasting blood specimens, while blood for the first series was drawn from two to three hours after breakfast.

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## SERUM PROTEINS IN PORTAL CIRRHOSIS

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THE characteristic changes in the serum proteins in cirrhosis of the liver are a fall in albumin and a rise in globulin, leading frequently to a reversal of the albumin/globulin ratio. The parts played by the euglobulin and pseudoglobulin fractions in this change in serum globulin are described and their significance discussed.

### MATERIAL

Cirrhosis of the liver is a common disease in Iraq. It is found principally, though not invariably, among the poor, and alcohol plays no part in its etiology. The course of the disease and the post-mortem findings resemble closely the Laennec's cirrhosis found in western countries. A detailed description of the condition has been given elsewhere.<sup>1</sup>

The thirty-six cases presented were male patients. The liver was small and ascites was present in all patients. Thus, in all, the disease was well developed. The difficulties in diagnosis in the early, pre-ascitic stage, particularly

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great in a country where other diseases affecting the liver are common, made unequivocal diagnosis desirable in a preliminary investigation. The Wassermann reaction was negative in twenty-eight and positive in eight of the patients.

Some difficulty was experienced in obtaining normal serum protein figures for comparison since the class concerned is commonly affected by gross malnutrition, chronic malaria, or conditions such as ankylostomiasis, leading to anemia. The results of estimations on normals are, therefore, presented in two groups, twelve apparently normal hospital patients and twelve medical students.

*Total protein* and *albumin* were estimated by the method of Greenberg,<sup>2</sup> and *euglobulin* by the following method derived from Proske and Watson.<sup>3</sup> Euglobulin was precipitated in a conical centrifuge tube by the addition of 0.2 c.c. serum to 6 c.c. 14 per cent sodium sulfate solution. After standing for twenty-four hours at 37° C., the tube was centrifuged, the precipitate washed twice with 3 c.c. portions of 14 per cent sodium sulfate, dissolved in 4.7 c.c. soda (1 c.c. 5 N. soda diluted to 23.5 c.c. with distilled water) and allowed to stand in boiling water for ten minutes. A standard was prepared in a 25 c.c. cylinder containing 2 c.c. standard tyrosine solution (0.20 Gm. tyrosine in 1,000 c.c. N/10 hydrochloric acid), 20.5 c.c. water and 1 c.c. 5 N. soda. The standard and the unknown were cooled to the same temperature in cold water and 0.3 c.c. Folin's phenol reagent added to the unknown and 1.5 c.c. to the standard. The volume of the unknown was made up to 5 c.c. and, after thorough mixing, compared in a colorimeter. In all the protein estimations, results were read from a curve constructed by reading a number of standard tyrosine solutions against a standard prepared as above.

The chromogenic power of euglobulin with Folin's phenol reagent was determined by sixteen parallel nitrogen estimations by micro-Kjeldahl. For calculating grams protein from grams N<sub>2</sub>, the customary factor, 6.25, was used, although no reports on the nitrogen content of euglobulin could be found. The results are shown in Table I. The factor for converting grams tyrosine into grams euglobulin is therefore 10.15, and there is a maximum error of 6 per cent. The difference between this factor and the factor given by Greenberg for globulin, namely 14.4<sup>4</sup> is caused by the use of heat in dissolving the euglobulin precipitate in soda.

TABLE I. THE CHROMOGENIC POWER OF EUGLOBULIN WITH FOLIN'S PHENOL REAGENT  
Column (a): Grams tyrosine colorimetrically equivalent to euglobulin present in 100 c.c. serum.  
Column (b): Grams euglobulin per 100 c.c. serum.

CASE	COLORIMETRIC	KJELDAHL	B/A
	(A)	(B)	
1	0.142	1.36	9.6
2	0.094	0.96	10.2
3	0.147	1.53	10.4
4	0.052	0.56	10.8
5	0.108	1.09	10.1
6	0.089	0.88	9.9
7	0.072	0.76	10.5
8	0.084	0.88	10.5
9	0.096	0.95	9.9
10	0.098	0.94	9.6
11	0.067	0.71	10.6
12	0.080	0.86	10.7
13	0.150	1.55	10.3
14	0.068	0.69	10.1
15	0.052	0.52	10.0
16	0.139	1.37	9.9
Average			10.15

From the estimations described, the quantities of the protein fractions were obtained by multiplying by the following factors: albumin, 16.6; globulin, 14.4; euglobulin, 10.15. Total protein was obtained by addition and pseudoglobulin by subtraction.

## RESULTS

The results are presented in Table II. Comparison of the two groups of normal subjects show that the better nutritional state of the students is reflected in the higher values of all protein fractions. The figures for the two groups of patients with cirrhosis are in close agreement. They can, therefore, be considered together since there is also no clinical reason for separating them.<sup>1</sup>

When the normal and cirrhotic subjects are compared, the fall in albumin, rise in globulin, and reversal of the A/G ratio is apparent. The euglobulin is

TABLE II. SERUM PROTEIN PARTITION IN NORMAL SUBJECTS AND IN PATIENTS WITH PORTAL CIRRHOSIS

NORMAL								CIRRHOSIS							
CASE	TOTAL PROTEIN (GM./100 C.C.)	ALBUMIN (GM./100 C.C.)	GLOBULIN (GM./100 C.C.)	EUGLOBULIN (GM./100 C.C.)	PSEUDOGLOBULIN (GM./100 C.C.)	ALBUMIN: GLOBULIN	EUGLOBULIN: ALBUMIN	CASE	TOTAL PROTEIN (GM./100 C.C.)	ALBUMIN (GM./100 C.C.)	GLOBULIN (GM./100 C.C.)	EUGLOBULIN (GM./100 C.C.)	PSEUDOGLOBULIN (GM./100 C.C.)	ALBUMIN: GLOBULIN	EUGLOBULIN: ALBUMIN
1	5.8	3.8	2.0	0.32	1.7	1.9	0.08	25	4.3	1.6	2.7	0.78	1.9	0.6	0.49
2	7.5	4.3	3.2	0.22	3.0	1.3	0.05	26	5.8	2.4	3.4	0.87	2.5	0.7	0.36
3	6.7	4.2	2.5	0.26	2.2	1.7	0.06	27	6.4	1.0	5.4	1.61	3.8	0.2	1.61
4	7.0	4.3	2.7	0.28	2.4	1.6	0.07	28	6.0	1.7	4.3	2.29	2.0	0.4	1.34
5	7.7	3.5	4.2	0.16	4.0	0.8	0.03	29	8.1	3.2	4.9	1.17	3.7	0.6	0.37
6	6.2	3.9	2.3	0.17	2.1	1.7	0.04	30	5.1	2.3	2.8	0.77	2.0	0.8	0.33
7	7.3	3.7	3.6	0.32	3.3	1.0	0.09	31	6.4	1.4	5.0	1.27	3.7	0.3	0.91
8	5.6	3.5	2.1	0.09	2.0	1.7	0.03	32	8.1	3.5	4.6	1.59	3.0	0.8	0.45
9	7.2	4.2	3.0	0.42	2.6	1.4	0.10	33	6.2	2.5	3.7	0.73	3.0	0.7	0.29
10	7.0	5.2	1.8	0.19	1.6	2.9	0.04	34	7.1	2.0	5.1	1.27	3.8	0.4	0.64
11	6.7	4.1	2.6	0.23	2.4	1.6	0.05	35	7.9	3.2	4.7	1.79	2.9	0.7	0.56
12	5.7	3.8	1.9	0.65	1.2	2.0	0.17	36	6.7	1.3	5.4	1.82	3.6	0.2	1.40
Av. (1 to 12)	6.70	4.04	2.66	0.28	2.38	1.52	0.07	37	5.5	1.9	3.6	1.21	2.4	0.5	0.64
13	7.4	4.8	2.6	0.26	2.3	1.8	0.05	38	7.8	2.7	5.1	2.24	2.9	0.5	0.83
14	8.1	5.0	3.1	0.53	2.6	1.6	0.10	39	7.7	3.2	4.5	1.64	2.9	0.7	0.51
15	6.9	3.6	3.3	0.20	3.1	1.1	0.06	40	7.1	2.7	4.4	1.41	3.0	0.6	0.52
16	8.4	5.5	2.9	0.18	2.7	1.9	0.03	41	6.5	1.9	4.6	1.73	2.9	0.4	0.91
17	8.2	5.1	3.1	0.55	2.5	1.6	0.10	42	7.1	2.5	4.6	1.81	2.8	0.5	0.72
18	8.3	5.0	3.3	0.36	2.9	1.5	0.07	43	6.5	1.6	4.9	1.98	2.9	0.3	1.24
19	6.7	4.3	2.4	0.21	2.2	1.8	0.05	44	5.4	1.2	4.2	1.73	2.5	0.3	1.44
20	6.9	4.5	2.4	0.37	2.0	1.9	0.08	45	6.6	2.3	4.3	1.26	3.0	0.5	0.55
21	7.6	4.6	3.0	0.39	2.6	1.5	0.08	46	8.3	2.7	5.6	2.02	3.6	0.5	0.75
22	6.0	3.8	2.2	0.49	1.7	1.7	0.13	47	6.7	2.2	4.5	1.01	3.5	0.5	0.46
23	8.2	5.5	2.7	0.43	2.3	2.0	0.08	48	7.5	1.9	5.6	1.54	4.1	0.3	0.81
24	8.2	5.1	3.1	0.59	2.5	1.6	0.12	49	6.1	2.2	3.9	0.75	3.1	0.6	0.34
Av. (13 to 24)	7.57	4.73	2.84	0.38	2.46	1.67	0.08	50	7.7	2.4	5.3	1.22	4.1	0.5	0.51
								51	6.2	2.0	4.2	1.07	3.1	0.5	0.54
								52	5.8	1.7	4.1	1.30	2.8	0.4	0.77
								53	7.1	2.0	5.1	1.97	3.1	0.4	0.99
								54	5.7	1.5	4.2	0.91	3.3	0.4	0.61
								55	6.4	2.8	3.6	1.02	2.6	0.8	0.36
								56	6.5	2.4	4.1	1.16	2.9	0.6	0.48
								57	6.9	2.6	4.3	1.74	2.6	0.6	0.67
								58	7.2	2.9	5.2	1.71	3.5	0.4	0.86
								59	7.8	3.5	4.3	0.92	3.4	0.8	0.26
								60	4.7	2.3	2.4	0.74	1.7	1.0	0.32
Av. (1 to 24)	7.14	4.39	2.75	0.33	2.42	1.60	0.08	Av. (25 to 60)	6.64	2.23	4.41	1.38	3.03	0.51	0.62

Cases 1 to 12, Normal patients.

Cases 13 to 24, Normal students.

Cases 25 to 52, Cirrhosis (Wassermann reaction negative).

Cases 53 to 60, Cirrhosis (Wassermann reaction positive).

considerably raised in the patients with cirrhosis, a point noticed by Gutman and Wise,<sup>5</sup> and in all subjects is outside the normal range; the average is four times the normal average. The range of pseudoglobulin values in the two groups is the same, although the averages show a small rise which is statistically significant. The calculation of the euglobulin/albumin ratio gives a figure which is, on the average, eight times as great as normal in cirrhosis.

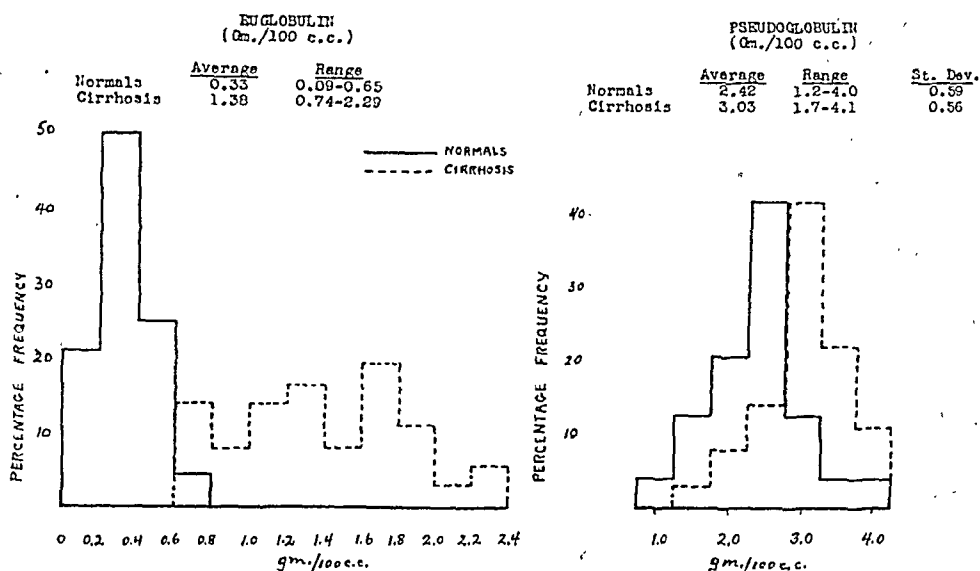


Fig. 1.

## DISCUSSION

The diet of the section of the community in which cirrhosis of the liver is most common in this country is deficient in protein, and patients with cirrhosis are averse to taking food containing much protein. Since animals on a low protein diet develop low serum albumin values,<sup>6</sup> it might be thought that the low serum albumin observed was dietetic in origin. However, four patients with cirrhosis, kept for two weeks on a high protein diet, showed no rise in serum albumin (Table III). Further experiments would be needed to ascertain if failure to absorb digested protein plays any part in the production of these changes.

The separation of serum proteins by salting out raises the question of the existence of these components as separate entities in serum. A change in the serum protein fractions in disease must reflect either a difference in the proteins themselves or the presence of substances that affect their precipitation reactions.

TABLE III. EFFECT OF A HIGH PROTEIN DIET ON THE SERUM PROTEINS OF FOUR PATIENTS WITH PORTAL CIRRHOSIS

CASE	TOTAL PROTEIN	ALBUMIN	GLOBULIN	EUGLOBULIN	PSEUDO-GLOBULIN
1(a)	6.8	2.2	4.6	1.3	3.3
(b)	6.3	1.7	4.6	1.9	2.7
2(a)	5.8	1.7	4.1	1.3	2.8
(b)	5.7	1.8	3.9	1.1	2.8
3(a)	5.5	1.8	3.7	1.6	2.1
(b)	5.2	1.3	3.8	1.8	2.0
4(a)	4.6	1.2	3.4	1.0	2.4
(b)	4.4	1.0	3.4	1.0	2.4

(a) On admission.

(b) After two weeks on high protein diet.

Investigations by electrophoresis and the failure of attempts to change the A/G ratio by "environmental disturbances" make the former more likely. The results presented here suggest that in cirrhosis a failure in the production of albumin is largely made good by increased euglobulin production. The serum euglobulin is known to be raised in a number of very different conditions, but no common factor has, as yet, been found. A search for other conditions in which these two fractions vary inversely might further elucidate the problem.

In the investigation of cases of cirrhosis of the liver, the estimation of serum euglobulin has advantages over the estimation of total globulin. Deviation from the normal is larger, and it might be expected to provide a more sensitive index of the abnormal protein metabolism of this disease. Moreover, the estimation is simple, and turbidometrically the measurement can be made very rapidly. However, a raised serum euglobulin should be used with caution as evidence of cirrhosis until more precise information is available as to the effect of liver diseases on euglobulin formation. Calculation of the euglobulin/albumin ratio gives an even greater deviation from normal but in the interpretation of this, other conditions leading to a lowering of the serum albumin must be excluded.

#### SUMMARY

1. Total protein, albumin, euglobulin, and pseudoglobulin were estimated in the serum of thirty-six patients with portal cirrhosis and the results compared with those of twenty-four normal individuals.

2. Serum euglobulin was above normal in all the patients with cirrhosis, the average value being four times the normal average.

3. Serum pseudoglobulin was within the normal range, but the average was raised; this rise was significant.

4. The serum euglobulin and the euglobulin/albumin ratio may provide more sensitive indices of disordered protein formation in this disease than the total globulin and the A/G ratio.

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## ANOXIA IN MALARIA

### AN EXPERIMENTAL STUDY ON DUCKS

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IT HAS been suggested that anoxia is an important factor in the mechanism of death in acute malaria. This theory was based upon a study of *Plasmodium falciparum* infection in a child,<sup>1</sup> *Plasmodium knowlsi* infections in monkeys, (2) and *Plasmodium lophurae* infections in ducks.<sup>3</sup> Recent studies have shown that transfusions will prolong the course of malaria in ducks but that they do not prevent the ultimate death of the birds from this disease.<sup>4</sup> The temporary improvement of the host following transfusions apparently results from a decrease in the anemia. Hill<sup>5</sup> concluded from her study on pigeons infected with *Plasmodium relictum* that death resulted from the anemia. Hewitt,<sup>6</sup> however, has stated that "No definite correlation has thus far been noted between the degree of anemia and fatal infections, except in the case noted above."

The relation of anemia produced by the malarial infection has been correlated in this study with the inability of the host to survive a diminution of oxygen pressure at simulated altitudes. It has also been observed that death is related to the severity of the anemia and is not directly related to the total number of parasites present at any time during the disease.

#### METHODS AND MATERIALS

*Plasmodium lophurae* was used in this study to infect young white Pekin ducks and young chicks obtained from a commercial hatchery. Parasites used for the inoculum were obtained from highly parasitized donor birds. The blood was mixed with an equal volume of a 2.0 per cent sodium citrate solution in physiologic saline. The parasitemia was followed by counting the number of parasitized cells per 500 red cells. Smears were stained with a combination of Giemsa and Wright stains. Standard methods were used in counting the red blood cells. Young erythrocytes were differentiated from adult red cells by the dark staining of the cytoplasm and the round nucleus.

Simulated high altitude was obtained by evacuating a tank of a capacity of 4 cubic feet with a vacuum pump. The rate of ascent and the levels of altitude were regulated by a needle valve through which a constant flow of air was admitted. The tank was equipped with a sight glass through which the birds were carefully observed during the entire experiment. Light was furnished by a 60-watt bulb located inside the chamber. The temperature of the interior was controlled by partially immersing the tank in a water bath and by permitting water to flow over the upper half. The average temperature was 28° C. The rate of ascent was 1,600 feet per minute during the first twelve minutes and then 600 feet per minute until either the desired altitude or the lethal altitude was reached.

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## EXPERIMENTAL

1. *A Comparison of the Lethal Simulated Altitudes for Normal and Malarial Infected Birds.*—In the first series of experiments a total of sixty-four normal chicks and thirty-seven chicks infected with malarial blood were placed in the decompression chamber from twenty-four to seventy-two hours following inoculation. They were decompressed at the rate given, until all the malaria-infected chicks were dead. In every instance the malaria-infected birds succumbed both at a lower altitude and following a shorter interval of exposure than did the normal controls. Normal birds died from anoxic anoxia with convulsions of a clonic type. The first symptoms of anoxia were sleepiness and inability to hold up the head. As the simulated altitude increased, the chick's head fell slowly to the floor, whereupon it was jerked erect. These movements were repeated. As the anoxia increased, the head was thrown rhythmically from side to side. This was the first sign of convulsions. If the bird was disturbed at this time, either by one of the other chicks or by movement, the final convulsions occurred immediately, while if the bird was undisturbed, the final convulsion did not occur for a minute or two. All deaths in normal and malaria-infected birds were the result of respiratory failure.

In a second series of experiments a total of twenty-six malaria-infected ducks and fifty-seven normal controls were placed in the decompression chamber. The behavior of the ducks was similar to that of the chicks with the exception that the symptoms of anoxia, convulsions, and death occurred at a higher simulated altitude in the ducks than in the chicks.

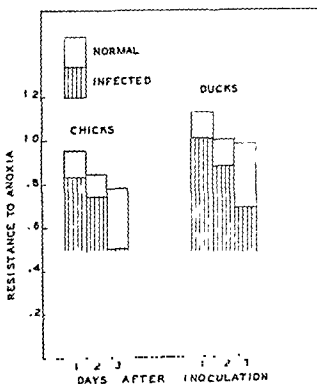


Fig. 1.—A comparison of the resistance to anoxia of malaria-infected and noninfected chicks and ducks. The resistance to anoxia, plotted on the ordinate, is calculated by multiplying the total altitude in feet times the total duration in minutes to which the bird was exposed to cause death.

The average results of these two series of experiments are shown in Fig. 1. The "resistance to anoxia," which is the product of the average simulated altitude at which convulsions and death occurred times the duration in minutes at this altitude, decreased with the daily progression of the infection. Since the experiments were terminated at the time of death of the last malaria-infected bird in the chamber, when approximately all the normal controls were

still alive, the level of resistance to high altitude of the normal birds only appears to fall on each successive day. Actually there was no appreciable decrease in resistance of the normals in this short interval. The parasitemia progressed rapidly from the third to the fifth day and death usually occurred by the sixth or seventh day of the disease.

2. *The Effect of Simulated Altitude on the Course of Malarial Infection in Birds.*—Normal ducks were decompressed to a simulated altitude of 20,000 feet and kept at this altitude for three or four days. They developed a polycythemia of 4 million red cells in comparison to the normal of 2.5 million. The peak of the polycythemia was reached in three days when the ducks were kept at a level of 20,000 feet.

In a third series of experiments, eight ducks were inoculated with malaria and the group was divided. One-half was placed in the decompression chamber and slowly decompressed to approximately 20,000 feet. The second half was kept at normal atmospheric pressure. Red cell counts, the number of young cells, the degree of parasitemia per 500 red cells, and the total number of parasitized cells per cubic millimeter were followed daily for five days in all of these birds. The average results obtained in this study are shown in Fig. 2.

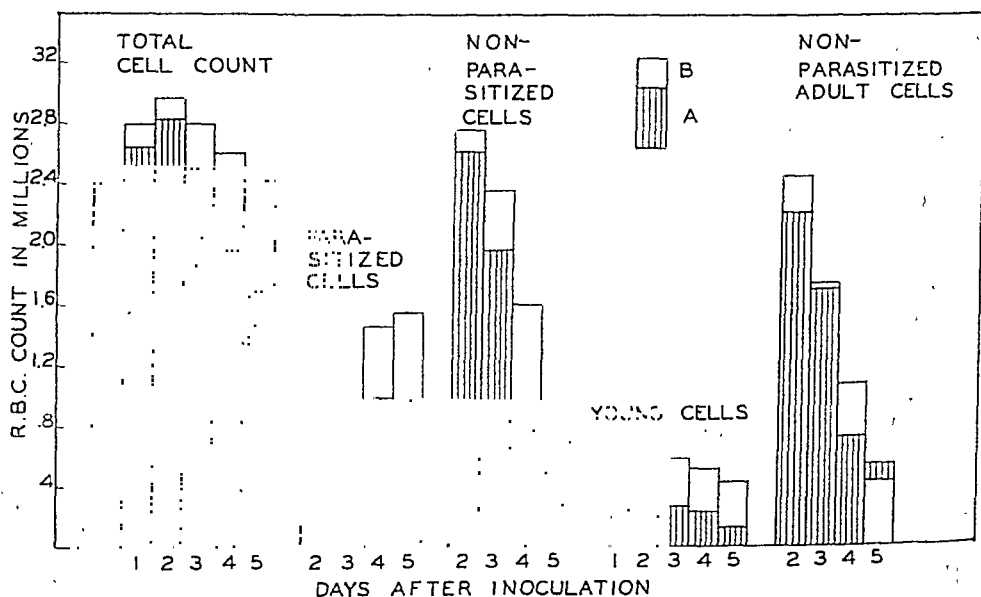


Fig. 2.—A comparison of total red blood cell counts, total parasitized cell counts, total nonparasitized cell counts, total young cell counts and the total nonparasitized adult cells of, A, a group of malaria-infected ducks kept at atmospheric pressure and, B, a group of malaria-infected ducks kept at simulated altitude of 20,000 feet for five days.

The total red cell count in Fig. 2 shows an increase on the second day and a subsequent fall thereafter in the birds kept in the chamber and also in those kept at normal atmospheric pressure. The rate of drop is considerably greater, however, in the latter group. The total number of parasitized cells is one-third greater in those birds kept at high altitudes when compared with those kept at normal atmospheric pressure. The total number of unparasitized cells decreases sharply in both groups as the disease progresses. The complete loss of these cells seems evident from the rate at which they disappear, as shown in Fig. 2. The formation of young cells is more rapid in the birds kept at high altitude. It will be noted from these data that the increase in number of the new cells in the

birds at simulated altitudes has not prevented the rapid disappearance of the old cells, and furthermore, these young cells cause the apparent slower rate of fall in the total number of remaining normal cells. It will be noted also that the rate of disappearance of adult erythrocytes is no greater in the birds at simulated altitude than it is in those birds kept at normal atmospheric pressure even though the total parasite count is higher in the former group.

Six ducks in another experiment were infected with malaria and four were taken to a simulated altitude of 20,000 feet. On the fifth day two of these birds were removed from the chamber. The total red cell count, the degree of parasitemia, and the total number of parasitized cells per eubic millimeter were followed in each of these ducks during the entire course of the disease.

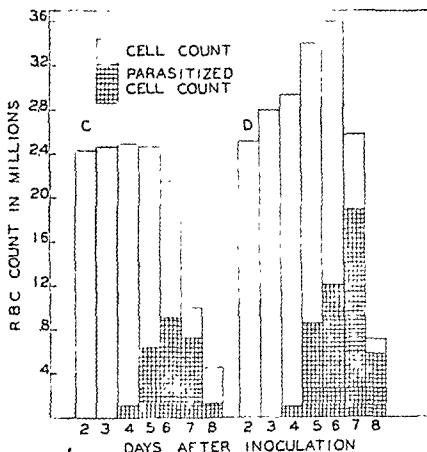


Fig. 2.—A comparison of the total red blood cell counts and total parasitized cell counts in, C, a malaria-infected duck kept at atmospheric pressure until death and, D, a malaria-infected duck kept at an altitude of 20,000 feet until death.

In Fig. 3 are shown the total number of red cells and the total number of parasitized cells in one of these ducks which died in the chamber at a simulated altitude of 20,000 feet and corresponding data on a control duck with malaria kept at atmospheric pressure which died on the same day. The malaria-infected bird kept at normal atmospheric pressure has less than half the total number of parasitized cells at the peak of the infection as compared with the bird kept at high altitude. Furthermore, one-third more red cells was destroyed in the bird kept at high altitude in the same length of time. The total number of parasitized red cells is approximately twice as great at the peak of the infection in the bird in the chamber as it is in the bird at atmospheric pressure. The total number of red cells in both birds falls to an extremely low level at the time of death. It may be as low as 200,000 or 300,000, or about one-tenth the normal count.

There were two ducks that survived the infection: one was kept at normal atmospheric pressure and the other was removed from the chamber on the fifth day after the inoculation. The data on these two birds are shown for comparison in Fig. 4. The number of parasitized red cells was somewhat higher in the bird

removed from the chamber. The level of the parasitized cell count at the depth of the anemia was approximately the same in the two birds and the rate of recovery was the same. The number of red cells destroyed between the peak of the infection and the time at which the smallest number of red cells was present is considerably greater in the bird which had been in the chamber than it is in the bird kept at atmospheric pressure.

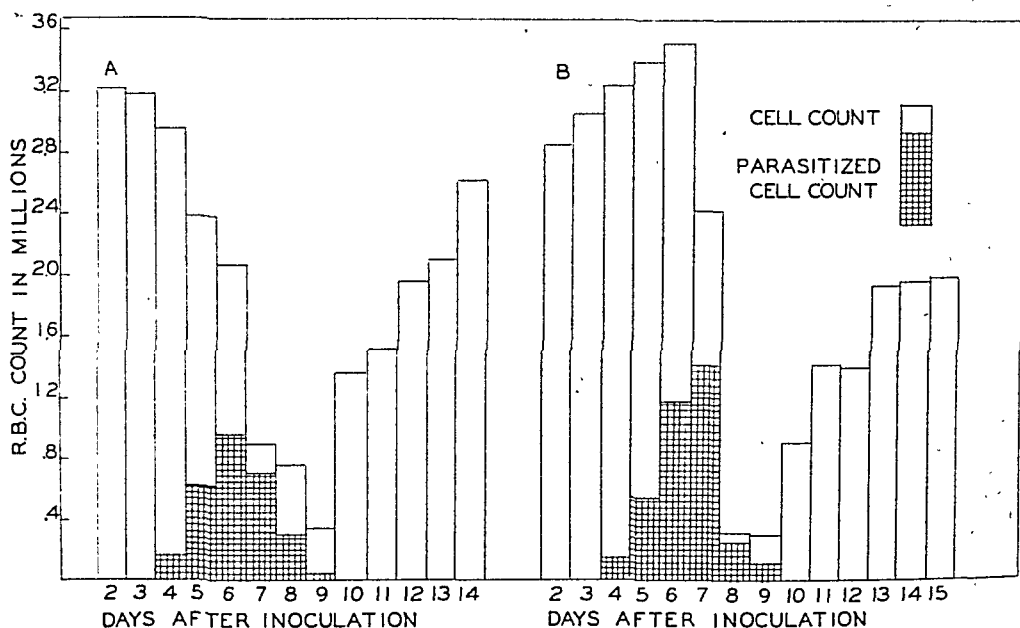


Fig. 4.—A comparison of the total red cell counts and the total parasitized cell counts of, A, a duck kept at normal atmospheric pressure and, B, a duck kept at an altitude of 20,000 feet for the first five days after the inoculation.

#### DISCUSSION

The results obtained in the first series of experiments indicate that birds with a mild malarial infection are definitely unable to withstand severe anoxia such as occurs at simulated high altitude. These birds were not obviously sick from the infection on the first, second, or third day after inoculation. The parasitemia was low on the third day in comparison with that at the peak of the infection. It is obvious, therefore, that ducks may show the effect of anemic anoxia before they reach the terminal stages of the infection. The sequence of the development of the symptoms of anoxia and the first indications of the convulsive state in the birds infected with malaria are identical to those occurring in birds not infected with malaria, except that the onset occurs in the former group at a lower altitude and after a shorter period of exposure. Ducks and chicks show the same sequence of development of symptoms to anoxia except that the first appearance of symptoms in the ducks occurs at higher altitude. It appears that the decrease in resistance to anoxia as observed in malaria-infected birds is the result of the inability of the oxygen transporting system to meet the increased demand accompanying the decrease in oxygen partial pressure.

The response of the erythrocyte-forming system in malaria-infected birds kept at normal atmospheric pressure is less than it is in infected birds kept at simulated altitudes. As a result of this earlier stimulation of the erythropoietic tissues the red blood cell count in the latter birds remains higher for a longer

period and the number of parasitized cells likewise increases. This increase in the number of parasitized cells may be explained by the fact that the parasites have a greater opportunity to parasitize red cells in the polycythemic birds. If the number of parasites decreases following the peak of the infection only as a result of phagocytosis by macrophages as recently stated by Taliaferro,<sup>7</sup> it would appear from our study that the phagocytosis of the parasitized cells is greatly diminished in the birds kept at high altitudes compared with birds kept at normal atmospheric pressure. The observation made by one of us that the course of the parasitemia in ducks infected with *P. lophurae* may be changed by transfusion with normal duck blood would suggest that factors other than immunologic ones play a significant role in the mechanism of the diminution in the number of parasites in this disease.<sup>4</sup> It also has been shown by McDougall<sup>8</sup> that the parasitemia in birds may be modified by inducing a state of hypoglycemia.

It would seem to us that the birds that survive a severe malarial infection may do so because the anemia develops slowly enough to cause a change in the environment that is unfavorable for the multiplication of the parasites before the anoxia resulting from the anemia becomes lethal for the host. If the decrease in the number of parasitized red cells were parallel to the decrease in the total red cell count, no bird would survive the infection. The fall in the number of parasitized cells is sharper than the fall in the number of red cells, as shown in Fig. 2.

The anemia in the chicks and ducks used in this study is extremely severe during the terminal stages of the disease. The great increase in the total number of parasitized cells in the birds kept at simulated altitudes did not produce an earlier death of the host. The failure of death to occur earlier in these birds would suggest that death in malaria is not dependent upon the total number of parasites present nor is it the result of a toxin produced by these parasites. It has been suggested that the plugging of capillaries by parasitized cells is an important factor in the mechanism of death in malaria.<sup>9</sup> It might be supposed that in the presence of an excessive number of both red cells and parasitized cells in the birds kept within the decompression chamber that capillary obstruction would occur earlier in the course of the disease. The plugging of capillaries by emboli, however, has not been observed in histologic studies made by one of us (R. H. R.) in cases of either malarial infection in man or experimental animals.

The data reported in this paper appear to lend support to the opinion that anoxia plays an important role in the mechanism of death in acute malarial infections.<sup>1-5</sup> The ducks in this study developed an anemia of such severity as to produce anemic anoxia preceding death. Furthermore, anoxia may be present in the malaria-infected ducks before the anemia becomes marked. This is indicated by the early death of the malaria-infected bird when placed at simulated high altitudes.

In considering the role of anoxia in acute malarial infections in man it should be remembered that the infection is accompanied by elevated temperatures and that the increment of oxygen utilization follows the normal  $Q_{10}$  for biologic reactions. The strain on the oxygen transporting system may be increased by the elevated temperature and contribute indirectly to the anoxia produced by the acute anemia.

## SUMMARY

1. The resistance of ducks and chicks infected with malaria to anoxic anoxia is less than that of normal birds.
2. The increase in susceptibility to anoxia increases as the malarial infection progresses.
3. Ducks with a polycythemia develop an extremely high total parasitized red cell count which may be double that occurring in malaria-infected birds kept at normal atmospheric pressure.
4. All birds develop a severe anemia preceding death. The total red cell count may reach a level of from 250,000 to 300,000.
5. It is suggested that the few birds in this study that survive the inoculum, fatal for the majority of ducks, may do so because the rate of decrease of the number of parasitized cells does not parallel the fall in the total red cell count during the terminal phase of the infection.
6. The experimental observations made in this study support the opinion that anoxia is a significant process in the mechanism of death in birds infected with *P. lophurae*.

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## ATTEMPTED TRANSMISSION OF HUMAN LEUCEMIA IN MAN

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THE problem of leucemia has been widely discussed in Forkner's monograph, *The Leukaemias and Allied Disorders*,<sup>1</sup> a book in which is summarized our knowledge of human and animal leucemia up to the year 1937. The advanced state of research into leucemia in mice and fowls, and the postulation of their principal identity with human leucemia because of clinical, symptomatologic, and morphologic analogies, makes it desirable to investigate the main features of leucemia in human beings. One important problem is that of the transmissibility of human leucemia. In mice and guinea pigs leucemia can readily

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be transplanted with cellular material in inbred strains, and it was in this way that the first difficulties of transmission were overcome. According to this experiment one would expect to have the best prospects of a take in man in transmission experiments in leucemic families or children of leucemic parents with leucemic material taken from a spontaneous case of leucemia of one of the members of the family or parents. Until now no family or children with leucemia have volunteered for such an observation and the studies to be described were conducted strictly on patients with a life prospect of two years or less. In mice, leucemia can be transmitted by intravenous, subcutaneous, or intramuscular injection of blood or other cellular leucemia material. In fowls leucemia can even be transmitted with cell-free material. In human beings there are only a few reports of intravenous injection of leucemia blood into other men.

Forkner mentions Schupfer<sup>2</sup> who injected, without result, the blood of a patient with chronic myeloid leucemia into three patients with carcinoma and also the blood of a man with acute leucemia into another patient with carcinoma. Moewes<sup>3</sup> attempted transmission with blood serum from a patient with leucemia into one with erythremia. Minot and Isaacs<sup>4</sup> transfused a patient with generalized lymphosarcomatosis with leucemic blood without result. Gamble<sup>5</sup> tried, without effect, to transmit lymphatic leucemia to a patient with myeloid leucemia by injecting 5 c.c. of blood intravenously. Gramen<sup>6</sup> accidentally used a donor for blood transfusion who a few days later developed acute leucemia; the recipient did not develop leucemia. R. Gosio<sup>7</sup> transfused into a patient with chronic lymphatic leucemia large quantities of blood from a patient with chronic myeloid leucemia with a high leucocyte count of 200,000 and 300,000, respectively. He studied the effect of the transfusion on the lymph nodules of the patient, taking biopsies at intervals. He reported proliferation of the histiocytes, increased reticulum fiber formation, and finally a tendency to scarring with repression of the leucemic infiltrations.

All these observations gave negative evidence without finally disproving the possibility of transmission. It was decided, therefore, to carry out similar investigations using a larger number of individuals.

#### ATTEMPTED TRANSMISSION TO PATIENTS WITH CARCINOMA AND OTHER CHRONIC DISEASES WITH A LIFE PROSPECT OF LESS THAN TWO YEARS

*Technique.*—Cellular suspensions of a lymph nodule or blood from the living patient or of suspension of splenic material (spleen removed post mortem) were injected subcutaneously. Ten grams of tissue from lymph nodule or spleen were ground with sterile sand and diluted with 30 c.c. of physiologic saline solution. The resulting suspension was spun for a few minutes at low speed and 2 c.c. of the supernatant emulsion containing numerous intact cells were injected subcutaneously into the forearm. The material used for injection was always fresh and was injected between twenty minutes and one hour after it had been taken from the patient. Post-mortem examinations were conducted immediately after death of leucemic patients and in cases of unavoidable delay in subsequent treatment, the spleen was kept at 4° C. after it was removed from the body. All material inoculated was bacteriologically sterile. The bloods of the recipients were examined before the inoculation of the leucemic material, daily afterward for a week, then periodically weekly, and after three months of observation, periodically monthly. Whenever possible a post-mortem examination was conducted on all patients inoculated.



So far, all results have been negative, possibly because most of our patients already had carcinoma, slight infection, etc. These negative results do not mean that leucemia is not transmissible; they show only that our trials were unsuccessful and that the technique and conditions should be altered.

The following are the protocols:

PROTOCOL 1.—Woman, 46 years of age. Acute myeloblastic leucemia. White blood cells on day of death, 800,000 cubic millimeter; hemoglobin, 2.9 Gm. per cent.

Fresh material from the spleen was injected into five patients within twenty minutes of the death of the leucemic patient, each receiving 2 c.c. subcutaneously in the forearm.

AGE (YR.)	SEX	SITE OF NEOPLASM	DEATH AFTER INOCULATION (DAYS)
59	M	Carcinoma of penis	26
58	M	Sarcoma of shoulder	13
43	F	Carcinoma of breast	39
45	F	Carcinoma of breast	88
82	F	Sarcoma of pelvis	31

Thick cellular suspension from the spleen kept for one hour at 4° C., 2 c.c., were injected subcutaneously into the forearm.

AGE (YR.)	SEX	NATURE OF DISEASE	DEATH AFTER INOCULATION (MO.)
76	F	Diabetes mellitus	28
73	F	Diabetes mellitus	24
38	F	Pernicious anemia	12
16	F	Aplastic anemia	8
53	F	Pernicious anemia	14

The spleen was kept at 4° C. overnight and was freshly ground up before injection; 2 c.c. of the cellular emulsion were injected subcutaneously into the left forearm.

AGE (YR.)	SEX	SITE OF LESIONS	DEATH AFTER INOCULATION	RESULT P.M.
33	F	Carcinoma of cervix	33 days	Negative
58	F	Carcinoma of bowel	30 days	Negative
45	F	Carcinoma of cervix	6 mo.	Negative
80	F	Carcinoma of bowel	26 mo.	Negative
41	F	Carcinoma of cervix	6 mo.	Negative
67	F	Carcinoma of cervix	5 mo.	Negative
77	F	Carcinoma of uterus	2 mo.	Negative
61	F	Carcinoma of breast	2 mo.	Negative
45	F	Carcinoma of cervix	6 mo.	Negative
68	F	Carcinoma of stomach	20 days	Negative
62	F	Carcinoma of pancreas	2 mo.	Negative
68	F	Carcinoma of breast	Still alive after 28 mo.	Negative
54	M	Carcinoma of skin	2 mo.	Results of blood and spleen ex- aminations en- tirely nega- tive; post- mortem exami- nation could not be per- formed
67	M	Carcinoma of stomach	6 mo.	
52	M	Carcinoma of stomach	1 mo.	
79	M	Carcinoma of skin	Still alive after 28 mo.	
61	M	Syphilis	Still alive after 28 mo.	
62	M	Carcinoma of rectum	7 mo.	
37	M	Carcinoma of bladder	4 mo.	

In all these cases clinically there was no increase in the leucocytes; regional lymph node and splenic enlargement did not occur.

PROTOCOL 2.—Man, 55 years of age. Subacute myeloid leucemia. White blood cells on day of death, 650,000 per cubic millimeter; hemoglobin, 4.2 Gm. per cent. Spleen removed five minutes after death; cellular emulsion inoculated twenty minutes after death. Two cubic centimeters subcutaneously injected into the forearm.

AGE (YR.)	SEX	NATURE OF DISEASE	DEATH AFTER INOCULATION	RESULTS
47	M	Heart failure	7 mo.	} Entirely negative
49	M	Heart failure	13 days	
51	M	Aneurysm of aorta	20 days	
68	F	Carcinoma of skin	Still alive after 28 mo.	
70	F	Carcinoma of skin	Still alive after 28 mo.	
66	M	Carcinoma of skin	Still alive after 28 mo.	
74	M	Carcinoma of tonsil	30 days	
88	M	Carcinoma of nose	23 mo.	

PROTOCOL 3.—Boy, 12 years of age. Acute myeloid leucemia. White blood cells on day of death, 1,200,000 per cubic millimeter; hemoglobin, 3.0 Gm. per cent. Spleen removed ten minutes after death; inoculation twenty-three minutes after death.

AGE (YR.)	SEX	NATURE OF DISEASE	RESULTS
67	F	Carcinoma of skin	} At post-mortem examination twenty-six months after inoculation no sign of leucemia
84	F	Carcinoma of skin	

PROTOCOL 4.—Woman, 52 years of age. Chronic myeloid leucemia with acute exacerbation. White blood cells, on day of death, 720,000 per cubic millimeter; hemoglobin 3.8 Gm. per cent. Spleen removed two minutes after death; inoculation eighteen minutes after death.

AGE (YR.)	SEX	NATURE OF DISEASE	RESULTS
60	M	Coronary occlusion	At post-mortem examination 14 months after inoculation no signs of a leucemia

In the following cases material from leucemic patients was injected intravenously.

PROTOCOL 5.—Blood from a patient with acute lymphatic leucemia, man, 37 years of age, blood Group A (II), with general enlargement of all lymph glands, tumor of the mediastinum and spleen, was injected intravenously into two patients with carcinoma of the esophagus. White blood cells on day of donation, 87,000 per cubic millimeter; hemoglobin, 6.7 Gm. per cent.

(1) Man, 55 years of age. Carcinoma of esophagus. Blood Group A (II).

(a) Injection of 50 c.c. of leucemic blood (white blood cells, 87,000).

(b) Injection eight days later of 80 c.c. of leucemic blood (white blood cells, 82,500). Death of patient occurred fourteen days after the first injection; at post-mortem examination no abnormality was detected. The daily blood examination until death and also the gross and microscopic necropsy examinations of the tissue were entirely negative.

(2) Man, 62 years of age. Carcinoma of esophagus. Blood Group A (II). Injection of 50 c.c. of leucemic blood (white blood cells, 82,000). Death of patient three days later. Daily blood examinations and post-mortem findings were negative.

PROTOCOL 6.—Woman, 36 years of age. Chronic myeloid leucemia. Blood Group B (III). White blood cells at time of injection, 279,000 per cubic millimeter; hemoglobin, 7.8 Gm. per cent. Intravenous injection of 50 c.c. of leucemic blood into a man, 60 years of age, Group B (III), suffering from coronary thrombosis with partial occlusion. Weekly blood examination did not show any abnormality for seven months. At post-mortem examination there was no evidence of leucemia.

PROTOCOL 7.—Man, 51 years of age. Chronic myeloid leucemia. Blood on the day of removal of lymph node showed white blood cells, 48,000 per cubic millimeter; hemoglobin, 3.43 Gm. per cent. An enlarged lymph node the size of an almond was removed with sterile precautions under local anesthesia. The node was ground up with sterile sand and saline. The mixture was spun at low speed and the supernatant cellular emulsion used for injections.

Two patients received 2 c.c. of the cellular emulsions subcutaneously in the forearm.

(1) Woman, 61 years of age. Carcinoma of the face.

(2) Woman, 82 years of age. Carcinoma of face.

There was no evidence of leucemia after thirty six and thirty-four months of observation.

TABLE I

PROTOCOL	SEX	AGE (YR.)	TYPE OF LEUCEMIA OF DONOR	DURATION OF LEUCEMIA IN PATIENT		INOCULATION -		BLOOD OF DONOR AT TIME OF INOCULATION								
				BEFORE INOCULATION	AFTER INOCULATION	MATERIAL USED	AMT. (C.C.)	Hb. (GM. %)	W.B.C. 10 <sup>3</sup>	POLYMORPHONUCLEARS	LYMPHOCYTES	EOSINOPHILES	BASOPHILES	MYELOCYTES	MYELOBLASTS	NUCLEATED REMS
1	F	46	Acute myeloblastic	6 days	—	Suspension of splenic material	2	2.9	800	1	—	—	—	2	97	—
2	M	55	Acute myeloblastic	122 days	—	Suspension of splenic material	2	4.2	650	8	—	—	2	10	77	3
3	M	12	Acute myeloblastic	2 days	—	Suspension of splenic material	2	3.0	1200	4	—	—	—	1	95	—
4	F	52	Chronic myeloid	13/12 mo.	—	Suspension of splenic material	2	3.8	720	16	—	4	1	3	72	4
5	M	37	Acute lymphatic	25 days	8 days	Blood	80 } 50 }	6.7	87	1	99	—	—	—	—	—
6	F	36	Chronic myeloid	14/12 mo.	20½/12 mo.	Blood	50	7.8	279	37	—	2	3	56	2	—
7	M	51	Chronic myeloid	21/12 mo.	½/12 mo.	Suspension of lymph nodule	2	3.43	48	2	—	4	6	12	70	6

## ATTEMPTED TRANSMISSION TO OTHER PATIENTS WITH LEUCEMIA

An attempt was made to transmit myeloid leucemia to patients with lymphatic leucemia and vice versa in order to see whether patients with one type of leucemia are susceptible to the other type.

*Technique.*—The blood of patients with chronic leucemia was used for transmission at a time when the white blood count was high, liver, spleen, and glands were generally enlarged, and just before treatment was necessary; the recipients were generally in a relatively good state of health, their chronic leucemia being reduced to a considerable degree by previous irradiation and arsenic therapy. This arrangement was adopted in the hope of enabling the respective transmitted cells to gain a footing in the body. Blood was transmitted only within the same blood group or with compatible blood groups. Both patients were brought together, and 6 c.c. of blood was taken extravenously from the florid leucemia with 0.6 c.c. of 2 per cent citrated saline; it was immediately injected intravenously into the recipient (within a minute after it was drawn). The full blood count of donor and recipient was done at the time of transmission, and both were examined weekly during the following months. All patients involved in the study were under continuous supervision for more than twelve months and had had previous full blood counts done at weekly intervals.

*Experiment.*—

1. Man, 36 years of age. Chronic myeloid leucemia for thirty-five months; white blood cells, 122,000 per cubic millimeter; hemoglobin, 7.9 Gm. per cent; polymorphonuclears, 27 per cent; myelocytes, 33 per cent; myeloblasts, 21 per cent; eosinophiles, 2 per cent; and basophiles, 16 per cent. This patient was used as a donor for a man, 45 years old, who had chronic lymphatic leucemia of thirteen months' duration; hemoglobin, 9.2 Gm. per cent; white blood cells,

11,000 per cubic millimeter; polymorphonuclears, 10 per cent; lymphoblasts, 90 per cent. The patient had just completed a course of deep x-ray therapy over spleen and thorax.

2. Woman, 36 years of age. Chronic myeloid leucemia for sixteen months. White blood cells, 95,000 per cubic millimeter; polymorphonuclears, 30 per cent; myelocytes, 41 per cent; myeloblasts, 11 per cent; eosinophiles, 1 per cent; basophiles, 14 per cent; nucleated reds, 3 per cent. This woman served as donor for a man, 53 years of age, with chronic lymphatic leucemia of thirteen months' duration. Hemoglobin, 10.64 Gm. per cent; white blood cells, 5,300 per cubic millimeter; polymorphonuclears, 54 per cent; lymphoblasts, 46 per cent. The patient had just completed a course of deep x-ray therapy two days previously.

3. Man, 63 years of age. Chronic lymphatic leucemia for twenty-six months. White blood cells, 90,500 per cubic millimeter; hemoglobin 10.82 Gm. per cent; polymorphonuclears, 7 per cent, lymphoblasts, 93 per cent. He served as a donor for two patients:

(a) Woman, 36 years of age. Chronic myeloid leucemia for 19 months. Hemoglobin 8.26 Gm. per cent; white blood cells, 51,500 per cubic millimeter; polymorphonuclears, 54 per cent, myelocytes, 31 per cent; myeloblasts, 7 per cent; eosinophiles, 1 per cent; and basophiles 7 per cent. The patient had just completed the last treatment of a deep x-ray course of therapy.

(b) Man, 42 years of age. Chronic myeloid leucemia for forty-eight months. Hemoglobin, 6.8 Gm. per cent; white blood cells, 68,000 per cubic millimeter; polymorphonuclears 62 per cent; myelocytes, 30 per cent; myeloblasts, 3 per cent; basophiles, 5 per cent. The patient had completed the last treatment of a deep x-ray course of therapy six days previously. He had slight palpitation and sweating three minutes after the transmission but walked home unaided afterward.

4. Man, 63 years of age. Chronic lymphatic leucemia for twenty-six months. Hemoglobin, 10.92 Gm. per cent, white blood cells, 95,000 per cubic millimeter; polymorphonuclears, 7 per cent; lymphoblasts, 93 per cent. This man served as a donor for a young woman, 32 years of age, who had had chronic myeloid leucemia for nineteen months. Hemoglobin, 9.5 Gm. per cent; white blood cells 28,000 per cubic millimeter; polymorphonuclears, 45 per cent; myelocytes, 41 per cent; myeloblasts, 1 per cent; eosinophiles, 6 per cent; and basophiles, 7 per cent. The patient had completed the latest course of irradiation therapy on the same day.

5. Man, 53 years of age. Chronic lymphatic leucemia for eleven months. Hemoglobin, 10.68 Gm. per cent; white blood cells 15,000 per cubic millimeter; polymorphonuclears, 15 per cent; lymphoblasts, 85 per cent. He served as a donor for a man, 36 years old, with chronic myeloid leucemia of thirty-six months' duration. Hemoglobin, 13.1 Gm. per cent; white blood cells, 13,500 per cubic millimeter; polymorphonuclears, 75 per cent; myelocytes, 5 per cent; myeloblasts, 1 per cent; basophiles, 16 per cent; lymphocytes, 2 per cent; and eosinophiles 2 per cent.

*Results of Investigation.*—Six months after the attempted transmissions, there was no evidence of success. Lymphatic leucemia did not develop in patients with myeloid leucemia, nor did myeloid leucemia develop in patients with lymphatic leucemia. Between forty and fifty days after the transmissions, all but one patient showed a flare-up of his respective type of leucemia without any apparent reason and without showing increased number of cells in the blood

characteristic of the transmitted type. This was remarkable in view of the fact that three of the patients had previously had rather regular intervals between the relapses of their chronic leucemia. The patients used to come in at three-, five-, and six-month intervals, respectively, for treatment, and now all came between the fortieth and fiftieth day after the experimental transmission. One would go too far in saying that the flare-up after fifty days was due to the transmission, because unexpected things happen always in leucemias, and common colds or other factors might have been responsible. Normal blood given in transfusion usually has the opposite effect and is frequently of beneficial effect in leucemia.

TABLE II

PROTOCOL	SEX	AGE (yr.)	DONOR'S LEUCEMIA	DONOR FOR RECIPIENT	AMOUNT OF BLOOD USED FOR TRANSMISSION (c.c.)	DONOR'S BLOOD AT TIME OF TRANSMISSION								DURATION OF DONOR'S LEUCEMIA IN RECIPIENT (MO.)				
						HEMOGLOBIN (gm. %)	W.B.C. 10 <sup>3</sup>	POLYMORPHONUCLEARS	LYMPHOCYTES	EOSINOPHILES	BASOPHILES	MYELOCYTES	MYELOBLASTS	NUCLEATED REDS	BEFORE INOCULATION	AFTER INOCULATION	TOTAL	
1	M	60	Chronic myeloid	1	6	7.9	122	27	-	2	16	33	21	1	35	7	42	
2	F	36	Chronic myeloid	2	6	8.2	95	30	-	1	14	41	11	3	16	13	29	
3	M	63	Chronic lymphatic	{3 4 5}	6.5	10.82	90.5	5	95	-	-	-	-	-	26	12	38	
4					6.5													
					6.5	10.92	95	7	93	-	-	-	-	-				
5	M	53	Chronic lymphatic	6	6	10.86	15	15	85	-	-	-	-	-	11	29	40 Still alive	

TABLE III

PROTOCOL	SEX	AGE (YR.)	RECIPIENT'S LEUCEMIA	DONOR'S LEUCEMIA	RECIPIENT'S BLOOD AT TIME OF INOCULATION								DURATION OF RECIPIENT'S LEUCEMIA (MO.)		
					HEMOGLOBIN (GM. %)	W.B.C. 103	POLYMORPHONUCLEARS	LYMPHOCYTES	EOSINOPHILES	BASOPHILES	MYELOCYTES	MYELOBLASTS	BEFORE INOCULATION	AFTER INOCULATION	TOTAL
1	M	45	Chronic lymphatic	Chronic myeloid	9.2	11	10	90	-	-	-	-	13	1 1/2	13 1/2
2	M	53	Chronic lymphatic	Chronic myeloid	10.64	5.3	54	46	-	-	-	-	13	27 1/2 Still alive	40 1/2
3	F	36	Chronic myeloid	Chronic lymphatic	8.26	51.5	54	-	1	7	31	7	19	11	30
4	M	42	Chronic myeloid	Chronic lymphatic	6.8	68	62	-	-	5	30	3	48	15	63
5	F	32	Chronic myeloid	Chronic lymphatic	9.5	28	45	-	6	7	41	1	19	15 1/2	34 1/2
6	M	36	Chronic myeloid	Chronic lymphatic	7.8	13.1	75	2	2	16	5	1	36	6 1/2	42 1/2

The fate of the recipients and donors is recorded in Tables II and III.

Donors and recipients had deep x-ray and arsenic therapy in the six months following the transmissions according to the state of their health or whenever necessary.

#### DISCUSSION

Whereas in fowls and mice leucemia can readily be transmitted in susceptible strains and only a minimal amount (one leucemic cell) of leucemic material is necessary for a successful transmission, all attempts of previous workers and the ones described herewith in man have failed. So far no transmission of leucemia from man to man, using blood, lymph nodule, or spleen, has been established. The reason for this failure might be sought in the following factors:

A. (1) *In the technique employed.* The assumed leucemic agent might also not be able to contact sufficient susceptible cells of its own type, that is, cells of the bone marrow or lymph nodules, by the subcutaneous or intravenous routes which were employed for the attempted transmissions. The agent of human leucemia might be extremely sensitive and not survive the procedure of transmission.

(2) *In the material used.* Whole blood, lymph nodules, and spleen might not be suitable for transmission in that they might either contain no leucemic agent or have it only in an inactive form.

(3) *Time of observation.* The time of observation in many cases was certainly very short, but even twenty-seven months might not be sufficient time for the agent to establish itself and produce a leucemia.

(4) *The choice of hosts.*

(a) As only people with a life prospect of under two years were available and all suffered from incurable diseases, the recipients could not be regarded as "virginal organisms." These patients might have had sufficient antibodies to prevent a take.

(b) The recipient might also have had a natural immunity, might have belonged to a nonsusceptible strain of man, or might have had an acquired immunity protecting him against any leucemic agent.

B. It also might be that human leucemia, contrary to animal leucemia, cannot be transmitted and therefore all attempts were useless from the start.

The fact that even material from acute leucemias would not produce reactions in the recipients is remarkable as one would expect that this type of leucemia with its fulminant course would be the easiest to transmit.

Of all the factors mentioned, the difficulty of obtaining suitable hosts and the apparently unsuitable routes are the most important and may certainly be responsible for the negative results of the transmission attempts.

#### SUMMARY

1. Attempts were made to transmit leucemia from man to man.
2. Material used consisted of blood and lymph nodular and splenic emulsions of chronic myeloid, lymphatic, and acute leucemias.
3. Attempts were made with whole blood to cross-transmit chronic myeloid leucemia to patients with chronic lymphatic leucemia and *vice versa*.
4. All transmission attempts from man to man and cross-transmissions between patients with leucemia failed with a period of observation of two years.

5. The explanation is regarded to be mainly due to unsuitable recipients and unsuitable route of transmission.

6. The studies brought forward no evidence that human leucemia is transmissible.

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## HEMATOLOGIC VALUES OF AMERICAN SOLDIERS STATIONED IN THE TROPICS

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THIS paper is the result of a survey of blood counts on American soldiers who have been stationed on tropical islands for twenty-four months. An unusual opportunity to study one group over a long period was afforded because the majority was from one task force.\* The survey was initiated because frequently medical officers would remark that "each month the red blood cell count seems to be lower and the incidence of eosinophilia increased." If this is true, then it would be a significant indication of the health of soldiers who have been garrisoned almost continuously on tropical islands since March, 1942.

#### MATERIAL AND METHODS

The material for this study was selected at random from the laboratory and clinical records of an Army hospital stationed on islands in the South and Southwest Pacific theaters of war since March, 1942. Data were collected from July, 1942 (six months after the group selected for study had left the continental limits of the United States), to January, 1944. The study was arbitrarily divided into three periods of six months each. Period I includes those studied from July, 1942, to January, 1943; Period II, from January, 1943, to July, 1943; and Period III, from July, 1943, to January, 1944. The same controls were utilized during the three periods. A comparable control group was studied in January, 1945, eleven months after the completion of this report.

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\*Two exceptions were (1) an organization that served one and one-half years in Panama and (2) one that served a year on another South Pacific island prior to joining the task force.

The same laboratory technicians performed the blood counts during the entire period of study. The Tallquist hemoglobin scale (15.8 Gm. of hemoglobin per 100 c.c. of blood) was the only method available for determining hemoglobin. An eosinophile count of 9 per cent or more was considered abnormal. All the patients had one or more stool examinations if eosinophilia was present.

The majority of all those studied lived in the northern United States prior to overseas duty. During Period I, all were garrisoned on a large base island in the South Pacific; during Period II, a large portion of patients studied were subjected to severe combat experience, during Period III, all studied were stationed on an isolated island in the Southwest Pacific but were not engaged in combat.

#### RESULTS

*Erythrocyte Counts and Hemoglobin Determination.*—During Period I (July, 1942, to January, 1943) the histories of 142 patients who had been stationed in the Tropics for six months were reviewed. The average erythrocyte count was 4,710,000, while the average hemoglobin determination was 88 per cent. Bacillary dysentery and acute infectious hepatitis were the main causes for admission.

During Period II (January, 1943, to July, 1943) the histories of 211 patients who had been stationed in the Tropics twelve months were reviewed. The average erythrocyte count was 4,560,000, and the average hemoglobin determination was 86 per cent. Respiratory diseases, bacillary dysentery, and malaria were the main causes for admission.

During Period III (July, 1943, to January, 1944) the histories of 302 patients who had been stationed in the Tropics eighteen months were reviewed. The average erythrocyte count of the group was 4,390,000, and the average hemoglobin determination was 84 per cent. Filariasis and malaria were the main causes for admission (Table I).

*Controls.*—A group of healthy soldiers selected from the hospital personnel, who suffered no illness requiring hospitalization during the period of observation, was utilized as controls. Complete blood counts are available on 14, made shortly after they arrived in the Tropics. The average erythrocyte count was 4,970,000, while the average hemoglobin was 95 per cent. Complete blood counts were performed on 19 others after they had been garrisoned in the South Pacific for one year. At that time the average erythrocyte count of the 33 was 4,770,000, while the hemoglobin average was 91 per cent. At the end of two years the average erythrocyte count of the 33 was 4,400,000, with a hemoglobin average of 84 per cent\* (Table II).

*Anemia.*—During Period I, 1 (0.7 per cent) patient of 142 studied was classified as having "borderline" normocytic anemia. No one in the control group was anemic. During Period II, 13 (6.1 per cent) of 211 patients studied were classified as having normocytic anemia. Similar findings were obtained in 2 (6 per cent) of 33 controls. In Period III, 29 (9.6 per cent) of 302 patients were classified as having normocytic anemia, while 3 (9 per cent) of 33 controls had similar findings (Table III).

*Leucocyte and Differential Counts.*—White blood cell and differential leucocyte counts in bacillary dysentery, acute infectious hepatitis, malaria, dengue,

\*At the end of three years in the tropics, a comparable but not identical group of controls had an average erythrocyte count of 4,700,000, while the hemoglobin average was 81 per cent. No one in the group was anemic.



TABLE I. AVERAGE ERYTHROCYTE COUNTS AND HEMOGLOBIN DETERMINATIONS ON PATIENTS STATIONED IN THE TROPICS FROM JULY, 1942, TO JANUARY, 1944

PERIOD	DISEASE	NUMBER OF CASES STUDIED	R.B.C. (IN MILLIONS)	HB. (%)	PER CENT* OF GROUP
Period I (Patients in Tropics from 6 to 12 months)	Dysentery, bacillary	23	4.69	86.9	16.2
	Peptic ulcer	8	4.68	88.8	5.6
	Hepatitis, infectious	28	4.96	88.2	20.0
	Neurosis (gastric, cardiac)	13	4.64	86.9	9.1
	Malaria, primary attack	13	4.49	86.1	9.1
	Respiratory (atypical pneumonia, bronchitis)	9	4.63	88.3	6.3
	Allergic (asthma, dermatoses)	11	4.72	88.5	7.7
	Arthritis	5	4.28	86.0	3.5
	Miscellaneous (including surgical cases)	26	4.78	92.1	18.3
	No disease found	6	4.45	82.5	4.2
	Summary	142	4.71 Av.	88.0 Av.	100.0
Period II (Patients in Tropics from 12 to 18 months)	Dysentery, bacillary	23	4.63	84.4	10.9
	Peptic ulcer	12	4.53	85.4	5.7
	Neurosis (gastric, cardiac)	14	4.66	87.5	6.6
	Malaria, recurrent	20	4.69	86.7	9.5
	Respiratory (atypical pneumonia, bronchitis)	29	4.64	86.7	13.7
	Allergic (asthma, dermatoses)	16	4.75	89.6	7.6
	Intestinal parasites (hookworm, whipworm)	10	3.89	81.5	4.7
	Dengue	4	4.70	86.2	2.0
	Renal (pyelo-nephritis, lithiasis)	16	4.55	80.9	7.6
	Miscellaneous (including surgical cases)	57	4.47	87.6	27.0
	No disease found	10	4.73	90.5	4.7
	Summary	211	4.56 Av.	86.0 Av.	100.0
Period III (Patients in Tropics from 18 to 24 months)	Dysentery, bacillary	2	4.50	82.5	.7
	Peptic ulcer	3	4.56	80.0	1.0
	Neurosis (gastric, cardiac)	11	4.30	81.8	3.6
	Malaria, primary attack	16	4.54	84.6	5.3
	Malaria, recurrent attack	13	4.42	85.0	4.3
	Respiratory (atypical pneumonia, bronchitis)	4	4.12	76.2	1.3
	Allergic (dermatoses, intestinal parasites)	9	4.27	78.8	3.0
	Filariasis	70	4.31	84.2	23.2
	Arthritis	2	4.20	80.0	.6
	Miscellaneous (including surgical cases)	124	4.46	84.8	41.1
	No disease found	48	4.35	82.9	15.9
	Summary	302	4.39 Av.	84.0 Av.	100.0

\*For statistical reasons average red blood cell counts and hemoglobin determinations for each period are calculated on a percentage basis.

dermatoses, intestinal helminthiasis, and filariasis—diseases commonly encountered in these theaters—did not differ from established values and are not presented.

*Eosinophilia.*—During Period I, of 142 patients studied, 1 (0.7 per cent) with hookworm disease had an eosinophile count of 9 per cent. During Period II, of 211 patients studied, 7 (3.3 per cent) had an eosinophile count of 9 per cent or more. Eosinophilia was present in two patients with hookworm disease and in one with periarteritis nodosa. It was unexplained in 4 patients. During Period III, of 256 patients studied, 26 (10.1 per cent) had an eosinophilia. Eosinophilia was present in patients with malaria, dermatoses, intestinal helminthiasis, and filariasis. Eosinophilia was unexplained in 9 patients (Table IV).

TABLE II. ERYTHROCYTE AND HEMOGLOBIN DETERMINATIONS ON HEALTHY SOLDIERS GARRISONED IN THE TROPICS OVER A PERIOD OF TWO YEARS

CASE	SIX MONTHS OR LESS IN TROPICS		ONE YEAR IN TROPICS		TWO YEARS IN TROPICS	
	R.B.C. (IN MILLIONS)	Hb. (PER CENT)	R.B.C. (IN MILLIONS)	Hb. (PER CENT)	R.B.C. (IN MILLIONS)	Hb. (PER CENT)
1	5.0	95	4.7	85	4.8	100
2	5.0	100	3.8*	65*	4.0	80
3	5.0	90	4.9	90	4.1	80
4	4.7	90	4.1	80	3.7*	80*
5	4.7	90	4.5	95	4.5	85
6	5.0	100	5.0	95	4.2	85
7	5.0	100	5.2	100	4.5	80
8	4.8	90	5.0	100	5.0	90
9	4.9	95	5.0	95	4.7	85
10	4.9	100	4.6	90	4.4	85
11	5.0	100	5.0	90	4.3	90
12	5.0	90	4.2	85	3.8*	70*
13	5.2	100	3.7*	65*	4.3	85
14	5.5	100	5.7	100	4.1	90
15			4.7	90	4.8	90
16			4.0	85	4.7	85
17			4.6	95	4.4	90
18			4.7	95	4.5	85
19			4.4	85	3.0*	70*
20			5.0	95	4.2	85
21			5.2	100	4.5	80
22			5.0	100	5.0	90
23			5.0	95	4.7	85
24			4.6	90	4.4	85
25			5.0	90	4.3	90
26			5.7	100	4.1	90
27			4.8	95	4.3*	75*
28			5.3	100	4.3	80
29			4.7	90	4.8	85
30			4.7	90	4.5	90
31			4.9	95	4.2*	75*
32			5.1	100	4.9	100
33			4.9	90	4.4	85
TOTAL			TOTAL		TOTAL	
14	4.97	95	33	4.77	91	33
						4.40
						84

\*Regarded as having an anemia. No organic reason could be found.

In order to clarify the higher incidence of eosinophilia during Period III, those studied during that period were divided into two groups: (A) those who had been garrisoned on filarial-free islands and (B) those who had been stationed for one year prior to joining the task force on an island where filariasis was endemic. (Previous observations on soldiers with filariasis<sup>1</sup> led us to expect a higher incidence of eosinophilia in that group.) Of 100 patients in Group A, 8 (8 per cent) had eosinophilia, while of 156 in Group B, 18 (11.5 per cent) had similar findings. Patients with eosinophilia in Group A had malaria, dermatoses, or hookworm infection, while in Group B, except for filariasis, eosinophilia could not be explained. These patients were admitted for inguinal hernia, impact molars, penile ulcer, acute infectious hepatitis, otitis externa, pharyngitis, cellulitis, and furunculosis.

Differential leucocyte counts on 282 healthy soldiers were utilized as controls. These men were also divided into two groups: (C) those garrisoned on filarial-free islands and (D) those exposed to filariasis. In Group C, 5 (3.5 per cent) of 140 had eosinophilia,\* while 40 (28.1 per cent) of 142 in Group D

\*During January, 1945, eleven months after leaving the island where the hospital was stationed during Period III, eosinophil counts were performed on 70 healthy soldiers, all from the original Group C. These men were all hospital personnel who had come overseas as a unit in March, 1942. They had been garrisoned almost continuously on tropical islands since that time. The study was important because it was now definitely known that the control group had lived in proximity to filariasis and had been exposed en masse to filariasis on another island in 1943.<sup>1</sup> Of 68 controls, 3 had hookworm disease, and 2 had eosinophilia of 9 per cent or more; while in 2 the eosinophilia was undiagnosed even after careful examination and did not have signs or symptoms of

TABLE III. ANEMIA IN SOLDIERS STATIONED IN THE TROPICS

PERIOD	PATIENTS				CONTROLS						
	TOTAL CASES	NUM- BER WITH ANEMIA	INCI- DENCE (PER CENT)	DISEASE	R.B.C. (IN MIL- LIONS)	HB. (PER CENT)	TOTAL CASES	NUM- BER WITH ANEMIA	INCI- DENCE (PER CENT)	R.B.C. (IN MIL- LIONS)	HB. (PER CENT)
I (July- Jan., 1943)	142	1	0.7	No disease found	4.0	70	14	0	0	--	--
II (Jan.- July, 1943)	211	13	6.1	Dysentery, bacillary	3.7	65	33	2	6	3.8	65
				Malaria, recurrent	3.5	60				3.9	65
				Amebiasis	4.1	65					
				Varicose veins	3.0	75					
				Hookworm	3.4	75					
				Hookworm	3.4	75					
				Hookworm	3.7	80					
				Hookworm	3.7	80					
				Dysentery, bacillary	3.7	65					
				Dysentery, bacillary	4.1	65					
				Neurasthenia	4.1	65					
				Malaria, recurrent	3.5	60					
				Appendicitis	3.7	65					
III (July, 1943- Jan., 1944)	302	29	9.6	Neurasthenia	3.6	75	33	3	9	3.7	80
				Neurasthenia	3.9	75				3.8	70
				Neurasthenia	3.6	75					
				Malaria, primary	3.9	70				3.9	70
				Malaria, recurrent	3.1	55					
				Malaria, recurrent	3.6	70					
				Malaria, recurrent	3.9	75					
				Dermatoses	3.9	70					
				Dermatoses	3.7	70					
				Dermatoses	3.8	75					
				Dermatoses	3.5	70					
				Respiratory	3.9	70					
				Respiratory	3.7	70					
				Respiratory	3.9	70					
				Anemia	3.7	75					
				Myositis	4.0	70					
				Otitis media	3.6	70					
				Hepatitis	3.9	75					
				Fracture	3.8	75					
				No disease found	3.6	75					
				No disease found	3.6	75					
				No disease found	4.1	60					
				No disease found	4.0	75					
				No disease found	3.6	75					
				No disease found	3.4	65					
				No disease found	3.7	75					
				No disease found	3.6	65					
				No disease found	3.8	75					
				No disease found	3.9	70					

TABLE IV. INCIDENCE OF EOSINOPHILIA (9 PER CENT OR MORE EOSINOPHILES) IN AMERICAN SOLDIERS IN THE TROPICS

PERIOD	TOTAL CASES	TOTAL CASES WITH EOSINOPHILIA	INCIDENCE EOSINOPHILIA (PER CENT)	DISEASE	NUMBER OF CASES	NUMBER OF CASES WITH EOSINOPHILIA
I	142	1	0.7	Hookworm	1	1
II	211	7	3.3	Periarteritis nodosa	1	1
				Hookworm	15	2
				Miscellaneous	18	4
III	256	26	10.1	Malaria, primary	16	2
				Malaria, recurrent	13	2
				Dermatoses	6	1
				Hookworm	3	2
				Filariasis	70	10
				Miscellaneous	148	9

had similar findings. In summation, 13 (5.4 per cent) of 240 soldiers (patients and controls) not exposed to filariasis had eosinophilia compared with 58 (19.4 per cent) of 298 who were exposed to the disease (Table V).

TABLE V. EOSINOPHILIA IN AMERICAN SOLDIERS EXPOSED TO FILARIASIS COMPARED TO THOSE NOT EXPOSED

PERIOD III				
GROUP	DISEASE	NUMBER OF CASES	NUMBER OF CASES WITH EOSINOPHILIA	INCIDENCE (PER CENT)
A. Patients never exposed to filariasis	Malaria, primary	16	2	
	Malaria, recurrent	13	2	
	Dermatoses	6	1	
	Hookworm disease	3	2	
	Miscellaneous*	62	1	
Total		100	8	8.0
B. Patients exposed to filariasis	Filariasis, clinical	70	10	
	Miscellaneous†	84	8	
Total		156	18	11.5
C. Healthy soldiers not exposed to filariasis		140	5	3.5
D. Healthy soldiers exposed to filariasis	No clinical signs	142	40	28.1
Total				
Those not exposed to filariasis		240	13	5.4
Those exposed to filariasis		298	58	19.4

\*No disease found.  
 †Inguinal hernia, impacted molars, penile ulcer, acute infectious hepatitis, otitis externa, pharyngitis, cellulitis, furunculosis.

#### DISCUSSION

It is generally believed that in the Tropics one may expect to develop anemia. The reasons usually ascribed are vague and the term "physiologic anemia" is used. Except for the high incidence of anemia due to parasitic infestations and dietary deficiency, the causes for this finding in indigent populations are similar to those which occur in persons living in a temperate climate. It is difficult to conceive that the causes for anemia in American soldiers in the Tropics would be comparable with indigents because of entirely different dietary and sanitary habits. Still, this report indicates that erythrocyte and hemoglobin values on soldiers who have been garrisoned continually in the Tropics over a two-year period are below the stated normal limits for young healthy males.<sup>2</sup> Although red blood cell counts are lower than normal, the actual incidence of anemia is negligible. It is important to determine if these findings are significant.

There are several possible explanations for lowered erythrocyte-hemoglobin values, such as chronic blood loss, iron deficiency, nutritional deficiency, alterations in the physiologic state of the body, or normal error in the erythrocyte count. There is increasing evidence that a diet deficient in iron does not produce iron-deficient anemia except in the presence of increased needs for iron, such as growth, pregnancy, or blood loss. In the Tropics iron-deficient anemia could be due to diminished or defective gastric secretions, but Heath<sup>3</sup> emphatically states that although inadequate iron absorption may be a complicating factor, blood loss in a subtle or chronic form can always be demonstrated. Malaria, amebiasis, hookworm disease, and the enteritides, diseases which represent a good portion of our study, are known to be associated with blood loss.

Lack of thiamine, riboflavin, and ascorbic acid may be contributing factors to defective blood formation.<sup>4</sup>

Statistically, the data accumulated on the patient group are not significant, since the number of cases studied is small and not identical in each period, and the severity of illnesses is not known to be comparable. It is assumed that the anemias reported in this group were secondary to the diseases. However, the data collected on the control group are significant. At the end of two years of tropical service, 18 (54.5 per cent) of 33 healthy soldiers had a substantial reduction in erythrocyte and hemoglobin values. These men were carefully studied, but no organic basis could be found for the low values. Rush<sup>5</sup> selected 14 of this group (Cases 1 to 14, Table II) and carefully studied their dietary habits. He found that the diet as served was adequate, but over 90 per cent of the group were eating meals inadequate in one or more essential food elements in that primary dietary deficiencies of calcium, thiamine, riboflavin, and ascorbic acid were revealed. On the other hand, iron and protein ingestion was adequate. All had weight loss during a six-month period (July, 1943, to January, 1944) and 12 had a reduction in red blood cells and hemoglobin. Specific causes for the loss or decrease in hematologic values were lacking except on a nutritional basis. Many possible factors seemed contributory. The most important perhaps was the monotony and lack of palatability of the food. Many complained of loss of energy, sleeplessness, and irritability (common complaints of soldiers in the Tropics) which could be explained by the continued threat of enemy bombing, heat and humidity of the jungle, isolation from civilization, and homesickness. At least one multivitamin tablet a day was added to the diet of these men, but there was no apparent increase either in weight or erythrocyte values during their stay in the jungle.

This same group of men moved to another island in March, 1944, where living conditions and food were much better. In addition, they all had a furlough to Australia. In a study of a comparable (but not identical) group in January, 1945 (thirty-six months of almost continuous tropical service), the average erythrocyte count was 4,700,000 and the average hemoglobin determination was 84 per cent. The majority had returned to normal weight, and their complaints of the previous year were long forgotten. However, although their erythrocyte counts approximated normal, the hemoglobin values did not change. It seems as though a multiplicity of secondary factors which contribute to poor nutrition may be one reason for the "low normal" erythrocyte values. Quinacrine hydrochlorida (atabrine) taken in suppressive doses since June, 1943, may be contributory. Physiologic changes that occur in the Tropics, such as those which increase blood volume, could be the prime factor. It has not been possible in this study to find any specific cause for our observations in healthy soldiers.

If the reasons postulated are true, then our data on patients gain more significance. As a broad general rule, soldiers have not developed anemia in diseases one commonly expects them to. Undoubtedly, early and specific therapy in malaria, helminthiasis, and gastrointestinal disorders minimize blood loss. This observation has been repeatedly checked during the past three years of tropical service. Many patients with these diseases have had several blood counts performed at intervals during their hospitalization. Although severe anemias have been found, especially in those with malaria due to *Plasmodium falciparum*, the data collected convinces one that anemias are not the rule. It seems plausible to conclude that the same factors affecting the control group

affect the patient group. More detailed study of larger control groups will be necessary before any accurate conclusions can be reached concerning specific causes for the observation reported in this paper.

The increasing incidence of eosinophilia presents an interesting problem because it is in the allergic and nonbacterial diseases that eosinophilia occurs. After troops have been stationed in the Tropics for two years, the incidence of eosinophilia (9 per cent or more) was 10.1 per cent compared with an incidence of 4.4 per cent in ambulatory patients studied at the Indianapolis City Hospital.<sup>7</sup> Data comparable with ours are reported by Allen,<sup>8</sup> who studied patients admitted to a Navy hospital in the South Pacific. He felt the increasing incidence was due to hookworm disease. The main reason for the increase of eosinophilia in our study was filariasis. This is best exemplified in the group of healthy soldiers studied during Period III (Table V). Although the group exposed to filariasis had no evidence of the disease, the incidence of eosinophilia was significantly increased over those not exposed.

Unexplained eosinophilia is not an infrequent finding in soldiers stationed in the Tropics. Allen<sup>8</sup> feels that in most instances, if the patient with eosinophilia is studied long enough, a parasitic infection is usually found. Because infestations are usually slight, manifestations may not appear for long periods of time, although eosinophilia, an allergic manifestation, develops early. Since Allen's report was read, 272 patients with an eosinophilia of 9 per cent or more have been carefully studied. Statistical data are not included in this report because groups are not comparable in that the majority spent less time in the Tropics and were garrisoned in Australia between combat missions. Although they were hospitalized for a wide variety of reasons (traumatic wounds, psychoneuroses, gastrointestinal complaints, arthritis, etc.), 55 were found to have helminthiasis, and in 52 others, symptoms were suggestive, but the patients were evacuated to rear areas before they could be studied adequately. Detailed data will be reported at a later date. In our control group of hospital personnel who had been in the Tropics thirty-six months, who have been garrisoned on an island where helminthiasis was hyperendemic in Periods I and II, and who were possibly exposed to filariasis during Period III, only 5 (7.3 per cent) had an eosinophilia, the causes for which were evident in 60 per cent.

#### SUMMARY AND CONCLUSIONS

1. Blood counts are reported on 655 American soldiers who were admitted to an Army hospital in the South and Southwest Pacific theaters of war.
2. The study was initiated after troops had been garrisoned in the Tropics at least six months and was divided into three six-month periods. Control groups of healthy soldiers were utilized.
3. A slight but progressive decrease below erythrocyte and hemoglobin values usually considered normal for young healthy males occurred. The incidence of true anemia was slight, even in those having diseases in which anemia is commonly found.
4. Possible reasons for these findings are discussed, but specific evidence for low normal values is lacking. Conditions which occurred on an isolated post in a forward area seemed to have played an important role.
5. A marked increase in the incidence of eosinophilia has been noted. The main causes are parasitic infestations and dermatoses.
6. A high incidence of eosinophilia was found in healthy soldiers who were exposed to filariasis but had no clinical evidence of the disease. This finding is

significant because it suggests that many soldiers are being exposed to diseases in which allergy may be an important factor. Because of minimal infestation, symptoms usually ascribed to these diseases may never become manifest.

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## LABORATORY METHODS

### THE INHIBITION ZONE TECHNIQUE FOR DETERMINATION OF BACTERIAL RESISTANCE TO PENICILLIN

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**P**ENICILLIN assay by the agar plate method is based on the principle that if a stipulated volume of penicillin is placed on the surface of an agar plate seeded with a susceptible organism, the zone (area) of growth inhibition will be a direct function of the concentration of penicillin employed. The cup methods of Abraham and co-workers<sup>1</sup> and of Foster and Woodruff,<sup>2</sup> the filter disk methods of Vincent and Vincent<sup>3</sup> and of Sherwood, Falco, and deBeer,<sup>3</sup> and the loop technique of Thomas, Levine, and Vitagliano<sup>4</sup> are examples of the application of this principle. In penicillin assay, the test organism and volume of penicillin solution applied to the surface of a seeded plate are maintained constant, the concentration of penicillin being the variable to be measured. In a previous communication we have pointed out that if both the volume and concentration of penicillin applied are maintained constant, while the organism employed as a seed is varied, the growth inhibition zone produced will be an inverse function of the resistance of the test organism. It was suggested that such a procedure would be more convenient and expeditious than the rather cumbersome serial dilution methods for evaluating bacterial resistance to penicillin. Responses to these suggestions indicate the desirability for elaboration upon the basis of the proposed technique and applications in the clinical laboratory.

#### RELATION OF RESISTANCE OF AN ORGANISM TO PENICILLIN IN BROTH AND ZONES OF GROWTH INHIBITION OF AGAR

For the purpose of the following report, resistance of test organisms (staphylococci) is expressed in terms of the minimum concentration of penicillin employed which prevented growth and the maximum concentration which permitted growth, in beef extract broth, after twenty-four hours at 37° C. Thus, if an organism grew in broth containing 0.02, but not if 0.04 Oxford unit of penicillin was present per cubic centimeter of medium, its resistance is expressed as 0.02 unit positive, 0.04 unit negative.

**Organisms.**—The cultures employed consisted of staphylococci obtained from suppurating infections and variants of the standard Oxford strain of *Staphylococcus aureus*, which were induced to develop increased resistance to penicillin, by serial transfer in broth containing progressively increasing concentrations of penicillin. (From a strain of *Staph. aureus* which did not grow in 0.04 Oxford unit per cubic centimeter, cultures which grew in broth containing 0.04 to 2.5 Oxford units per cubic centimeter were obtained after from two to fifteen test-tube generations.)

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**Penicillin.**—The penicillin solutions employed for this study were those encountered in routine clinical work diluted to make concentrations of 10, 5, 2.5, 1.25, and 0.63 Oxford units per cubic centimeter in phosphate buffer (M/50 at pH 7.2). All of these penicillin samples, when diluted serially in broth, markedly inhibited growth of the Oxford strain of *Staph. aureus* in a concentration of 0.02 Oxford unit per cubic centimeter of medium, as did also a secondary penicillin standard. When 4 mm. loopfuls of these penicillin solutions containing 10 Oxford units per cubic centimeter were placed on agar plates seeded with the Oxford strain of *Staph. aureus*, growth inhibition zones of  $29 \pm 1$  mm. in diameter were produced. Comparison with a secondary penicillin standard \* disclosed that all samples were within limits of error inherent in penicillin assays and the leeway ( $\pm 15$  per cent) permitted in army specifications.

**Preparation of Plates.**—Seeded agar plates were prepared by inoculating 0.05 c.c. of a 20 to twenty-four hour broth culture of a test organism into 15 c.c. of melted, cooled (from 42 to 48° C.) agar which was then poured into a Petri dish which, immediately after hardening, was placed in the refrigerator (from 5 to 10° C.) where it was kept until ready for use (from two to eight hours).

**Determination of Growth Inhibition Zones.**—A 4 mm. loopful of each of the aforementioned concentrations of penicillin was placed on the surface of a chilled agar plate which had been seeded with staphylococci of various degrees of resistance (as determined by growth in broth containing known concentrations of penicillin), and the plates were immediately placed in the incubator at 37° C., taking care to maintain the plates horizontal in order to insure development of circular growth inhibition zones. After from eighteen to twenty-two hours' incubation, the diameters of the growth inhibition zones were measured to the nearest 0.5 mm.

**Determination of Inhibiting Concentration of Penicillin in Broth.**—Beef extract broth containing various concentrations of penicillin (2.5, 1.25, 0.63, 0.32, 0.16, 0.08, 0.04, 0.02, and none) was distributed aseptically into test tubes (10 c.c. quantities). A tube of each concentration was inoculated with a drop (0.05 c.c.) of a (twenty to twenty-four hours at 37° C.) broth culture of the test organism. The inoculated tubes were then placed at 37° C. and vigor of growth recorded after from eighteen to twenty-two hours' incubation.

TABLE I. GROWTH INHIBITION ZONES PRODUCED BY VARIOUS CONCENTRATIONS OF PENICILLIN ON AGAR SEEDED WITH STRAINS OF STAPHYLOCOCCI OF VARYING DEGREES OF RESISTANCE TO PENICILLIN IN BROTH

GROUP	NUMBER OF STRAINS	CONCENTRATION OF PENICILLIN IN BROTH (OXFORD UNITS PER C.C.)		CONCENTRATION OF PENICILLIN APPLIED (4 MM. LOOPFULS) TO SEEDED AGAR (OXFORD UNITS PER C.C.)				
		PERMITTING GROWTH (24 HR.)	PREVENTING GROWTH (24 HR.)	10	5	2.5	1.25	0.63
				ZONE OF GROWTH INHIBITION (DIAMETER IN MM.)				
A	16	0.02	0.04	$28.9 \pm 1.2$	$24.6 \pm 0.8$	$20.4 \pm 1.1$	$15.0 \pm 2.5$	$10.5 \pm 2.9$
B	9	0.04	0.08	$24.6 \pm 1.3$	$21.3 \pm 1.2$	$16.9 \pm 1.3$	$12.0 \pm 2.0$	$< 5^*$
C	7	0.08	0.16	$18.9 \pm 1.0$	$15.2 \pm 1.1$	$11.6 \pm 1.3$	$< 5^*$	
D	4	0.16	0.32	$17.1 \pm 0.3$	$12.3 \pm 0.9$	$< 5^*$		
E	5	0.32	0.63	$14.0 \pm 0.8$	$9.9 \pm 0.7$			

\*Inhibition zone not distinct.

\*Supplied by Merck & Co. Inc., Rahway, N. J.

*Comparison of Inhibiting Concentration in Broth With Inhibition Zone on Agar.*—The results obtained with the foregoing techniques for measuring bacterial resistance to penicillin are summarized in Table I, where (1) the test organisms are allocated to five groups on the basis of the maximum concentration of penicillin permitting and the minimum concentration preventing growth in broth and (2) the diameters of the growth inhibition zones (with their standard deviations) obtained on agar seeded with organisms of the respective groups are shown.

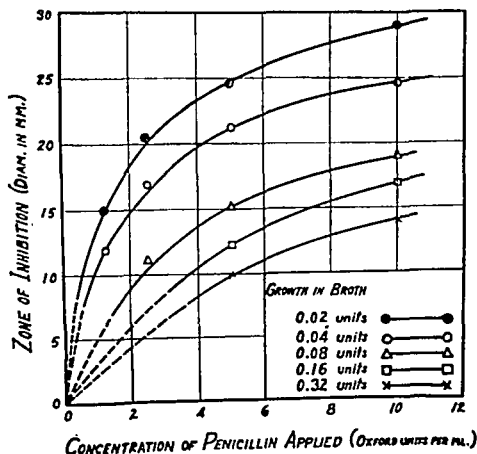


Fig. 1.—Relation of concentration of penicillin to inhibition zones produced against staphylococci of various degrees of resistance to penicillin in broth.

By plotting the inhibition zones against the concentrations of penicillin applied, the curves shown in Fig. 1 are obtained. It will be noted from Table I that when concentrations of penicillin of less than 2.5 Oxford units per cubic centimeter were used, the diameters of inhibition zones obtained showed a rather high degree of variability, a phenomenon which is generally the case for inhibition zones of less than 12 mm. diameter. It will be further noted that when employing a penicillin solution containing 2.5 Oxford units per cubic centimeter, the inhibition zones produced with organisms of group D (growth in broth containing 0.16 but not if 0.32 Oxford unit of penicillin per cubic centimeter is present) which are probably at the upper limit of susceptibility with present methods of systemic penicillin treatment, the inhibition zones are too small for satisfactory evaluation. Even for organisms of group C (growth in broth with 0.08 but not 0.16 Oxford unit of penicillin per cubic centimeter), which are quite susceptible, the inhibition zones produced with a loop of penicillin solution containing 2.5 Oxford units per cubic centimeter are in the range of minimum reliability. It is therefore suggested that penicillin solutions containing 10 or 5 Oxford units per cubic centimeter would be most useful for rapidly determining penicillin sensitivity by the method of ascertaining the growth inhibition zones produced on agar plates seeded with a test organism.

If the growth inhibition zones obtained for the various groups of test organisms (employing a standard 4 mm. loop of 10 or 5 Oxford units per cubic centimeter of buffered penicillin solution) are plotted against the resistance of the respective groups as shown by growth in broth with various concentrations of penicillin (for example, the maximum concentrations of penicillin employed which permitted, and the minimum concentrations which prevented, growth in broth), the curves in Fig. 2 are obtained. Table II was then developed from these curves (Fig. 2) and shows the probable concentrations of penicillin (expressed in Oxford units per cubic centimeter) which would permit or prevent growth in broth, for designated inhibition zones obtained on an agar plate seeded with a test organism.

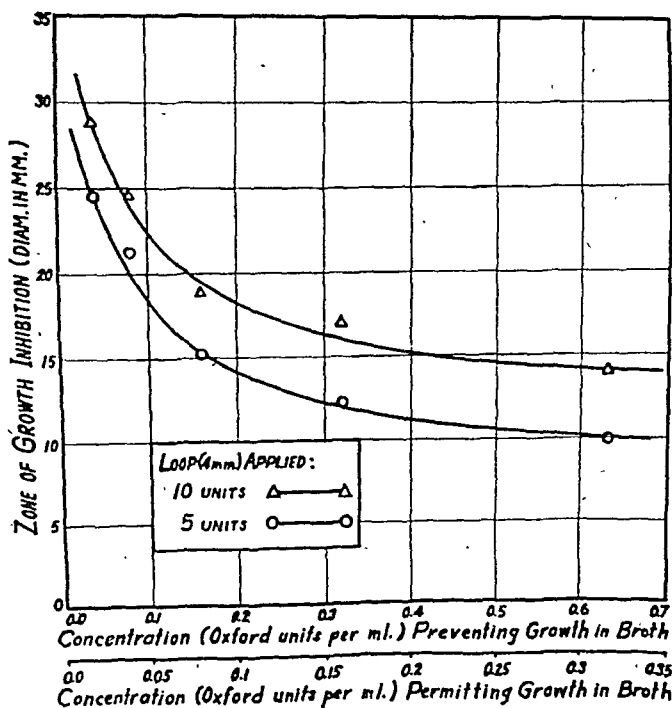


Fig. 2.—Relation of inhibition zones on seeded agar to resistance of staphylococci to penicillin in broth.

In practice, knowledge of the exact degree of resistance of an organism to penicillin is not so significant as the information as to whether a suspected organism is so susceptible (or resistant) that the concentration of penicillin maintainable in the blood stream may reasonably be expected to be effective (or ineffective) against the organism in question. A concentration of 0.25 Oxford unit per cubic centimeter of blood is maintainable by the intravenous drip and intramuscular administration of penicillin. Assuming that an organism which grows in broth containing 0.2 Oxford unit, but not in 0.4 Oxford unit, of penicillin per cubic centimeter is near the limit of bacterial resistance amenable to the present available methods for systemic treatment with penicillin, then, if a 4 mm. loopful of a solution containing 10 Oxford units of penicillin per cubic centimeter were applied to a plate seeded with the organism in question, the growth inhibition zone produced would be 15 mm. in diameter. (If the penicillin solution employed for the test contained 5 Oxford units per cubic centimeter, an inhibition zone of 11 mm. would be obtained [see Table II].)

TABLE II. RELATION OF GROWTH INHIBITION ZONE ON AGAR SEEDED WITH STAPHYLOCOCCI TO PROBABLE CONCENTRATION OF PENICILLIN PERMITTING AND PREVENTING GROWTH IN BROTH

10 OXFORD UNITS PER C.C. (4 MM. LOOPFULS APPLIED TO SURFACE OF SEEDED AGAR)			5 OXFORD UNITS PER C.C. (4 MM. LOOPFULS APPLIED TO SURFACE OF SEEDED AGAR)		
ZONE OF GROWTH INHIBITION ON SEEDED AGAR PLATE (IN MM.)	PROBABLE CONCENTRATIONS* IN BEEF EXTRACT BROTH (OXFORD UNITS PER C.C.)		ZONE OF GROWTH INHIBITION ON SEEDED AGAR PLATE (IN MM.)	PROBABLE CONCENTRATIONS* IN BEEF EXTRACT BROTH (OXFORD UNITS PER C.C.)	
	PERMITTING GROWTH	PREVENTING GROWTH		PERMITTING GROWTH	PREVENTING GROWTH
30	0.02	0.03	28	0.01	0.02
29	0.02	0.04	27	0.02	0.03
28	0.03	0.05	26	0.02	0.03
27	0.03	0.05	25	0.02	0.04
26	0.03	0.06	24	0.03	0.05
25	0.04	0.07	23	0.03	0.06
24	0.04	0.08	22	0.03	0.06
23	0.05	0.10	21	0.04	0.07
22	0.06	0.12	20	0.04	0.08
21	0.07	0.13	19	0.05	0.09
20	0.09	0.15	18	0.06	0.11
19	0.08	0.18	17	0.06	0.12
18	0.11	0.22	16	0.07	0.14
17	0.14	0.27	15	0.09	0.17
16	0.16	0.32	14	0.11	0.21
15	0.21	0.42	13	0.13	0.25
14	0.33	0.65	12	0.16	0.32
13	....	....	11	0.21	0.42
12	....	....	10	0.32	0.63
11	....	....			
10	....	....			

\*To nearest 0.01 unit.

Our experience has indicated that for streptococci (and other bacteria which do not grow readily on plain agar but grow well on blood media), determination of growth inhibition zones on seeded blood agar plates yielded very satisfactory results. When employing such plates with hemolytic (alpha and beta) streptococci or staphylococci, the area of growth inhibition is indicated by a zone of unhemolyzed red cells surrounded by a narrow clear hemolyzed zone due to diffusion of hemolytic agents from the vicinity of bacterial growth. With the strains of pneumococci encountered, and employing a heavy inoculum, the growth inhibition zone consisted of a series of concentrically ringed bands producing a rainbowlike effect—an area of unhemolyzed red cells surrounded by a rather wide zone of light greening and hemolysis which was, in turn, circumscribed by a narrow zone of what appeared to be heavy growth. With a light inoculum this rainbowlike effect was not manifest.

In general, if the growth inhibition zone produced by applying a 4 mm. loopful of a penicillin solution containing 10 Oxford units per cubic centimeter to the surface of a seeded agar plate is less than 10 mm. in diameter, the test organism would be considered too resistant to anticipate satisfactory results; inhibition zones of from 10 to 15 mm. in diameter would place the organism in a questionable category regarding amenability to systemic penicillin therapy; organisms showing inhibition zones greater than 15 mm. in diameter would be considered sufficiently susceptible to warrant expectation of satisfactory results with the present methods of systemic penicillin therapy. If, in the future, higher penicillin blood levels can be maintained than are now feasible, it will merely be necessary to increase, appropriately, the concentration of the test solution (10 Oxford units per cubic centimeter as now recommended) to adapt the proposed method to the new conditions.

The inhibition zone technique described herein for determination of bacterial resistance to penicillin is subject to less error, requires no special apparatus and considerably less media and equipment, and is not subject to the hazard of contamination associated with the various dilution techniques; the probable degree of resistance can, if necessary, be tentatively determined within eight to ten hours after application of penicillin to seeded plates.

#### PROCEDURE FOR DETERMINATION OF BACTERIAL RESISTANCE TO PENICILLIN BY THE LOOP GROWTH INHIBITION ZONE TECHNIQUE

##### A. Materials needed

1. Broth (or other liquid medium) culture of test organism (eighteen to twenty-four hours at 37° C.)
2. Melted agar (15 c.c.) cooled to from 42 to 48° C.
3. One sterile Petri dish (marked off in quadrants)
4. Pipettes—1, 5, and 10 c.c. capacity
5. Standard 4 mm. loop (preferably platinum)
6. M/50 phosphate buffer at pH 7.2 (may be prepared by adding 27 c.c.  $\text{KH}_2\text{PO}_4$  solution containing 2.72 Gm. per liter to 73 c.c.  $\text{Na}_2\text{HPO}_4$  solution containing 2.84 Gm. per liter)
7. Penicillin solution (10 and/or 5 Oxford units per cubic centimeter in M/50 phosphate buffer)
8. Sterile flasks (150 c.c.) and test tubes (about 18 mm. diameter)

##### B. Procedure

###### 1. Preparation of penicillin solutions

- (a) Place 1 c.c. of penicillin (as prepared for therapeutic use and presumably containing 5,000 Oxford units per cubic centimeter) into 49 c.c. of the phosphate buffer (this will make a solution of 100 Oxford units per cubic centimeter).
- (b) Add 1 c.c. of this diluted penicillin to 9 c.c. phosphate buffer to prepare a solution containing 10 Oxford units per cubic centimeter. (If desired, a solution of 5 Oxford units may be prepared by mixing equal volumes of this solution with phosphate buffer.)

Note: In view of the error inherent in penicillin assay and unavailability of penicillin standards for clinical laboratories, penicillin (5,000 Oxford units per cubic centimeter) solutions employed (diluted 1:500 for 10 or 1:1000 for 5 Oxford units per cubic centimeter) should be tested as described below against the Oxford strain of *Staph. aureus* (or other suitable organism). A growth inhibition zone of  $29 \pm 1$  mm. in diameter with a dilution of 10 Oxford units (or  $25 \pm 1$  mm. with that of 5 Oxford units) may be taken to indicate that the penicillin solution is within the limits of concentration usable for determination of bacterial resistance.

###### 2. Preparation of seeded agar plates

Add 0.05 c.c. (or one drop from a 1 c.c. pipette) of a broth culture (from 20 to twenty-four hours at 37° C.) of the test organism (*Staphylococcus*\*) to 15 c.c. of melted, cooled (from 42 to 48° C.) agar. Roll between the palms of the hands to distribute the inoculum (but avoid bubbles if possible) and pour into a Petri dish. Mix, permit to solid-

\*For streptococci (or other organisms which do not grow readily on nutrient agar but grow luxuriantly on blood media), add from 0.5 to 0.75 c.c. of citrated blood before introducing the test organism, and it may be desirable to increase the size of the inoculum to 0.1 c.c. of a liquid medium in which the test organism grows well.

ify, and place in the refrigerator just as soon after solidification as is feasible. (Seeded plates prepared in this manner may be used after from two to eight hours' cooling, and plates kept in the refrigerator for twenty-four hours have given satisfactory results.)

### 3. Determination of resistance (growth inhibition zone).

(a) Remove the cooled, seeded agar plate from the refrigerator and

- (1) Wipe off any moisture appearing on the inner surface of the Petri dish lid with a clean towel or cloth.

Note: There were no difficulties due to moisture accumulating on the upper lid of the Petri dish, when maintained right side up in water-jacketed incubators, and only three instances of inconsequential contamination have been noted among over 4,000 bacterial resistance tests, when the dishes were wiped as described above.

- (2) Mark off the bottom of the plate into four sectors (if not already so marked) and label for identification.
- (b) Place a 4 mm. loopful of a penicillin solution containing 10 Oxford units per cubic centimeter on each of two or more of the sectors. (The practice in this laboratory has been to employ solutions of both 10 and 5 Oxford units per cubic centimeter of penicillin, placing a loop of each of these on each of two quadrants, to provide a check on both the concentration and constancy of loopfuls.)

Note: In our experience, employing a loop (bent at right angles to the wire) inserted into the penicillin solution (preferably in a tube not less than 18 mm. diameter), maintaining the plane of the loop approximately parallel to the surface of the liquid and quickly withdrawing it, in order to obtain a bead of liquid, was found to give consistent results.

- (c) To check the penicillin, 4 mm. loopfuls of the solutions of 10 and/or 5 Oxford units per cubic centimeter (1:500 or 1:1000 dilutions, respectively, of 5,000 Oxford units employed therapeutically) should be placed on agar seeded with the Oxford strain of *Staph. aureus*. A growth inhibition zone of  $29 \pm 1$  mm. in diameter with the dilution of 10 Oxford units per cubic centimeter (or  $25 \pm 1$  mm. in diameter with that of 5 Oxford units per cubic centimeter) indicates that the penicillin solution being employed is of suitable concentration.
- (d) Place the seeded Petri dish to which the loopfuls of penicillin have been added into the incubator (*right side up*), taking care to see that the bottom of the dish is horizontal in order to insure circular zones of growth inhibition.

### C. Examination and record

1. After incubation overnight (from eighteen to twenty-two hours), measure the diameters of the growth inhibition zones to the nearest 0.5 mm. (This may be done by placing the Petri dish on a plate counter and slipping a thin rule graduated in millimeters under it, etching a scale on the plate counter, or merely holding up the plate against a light and measuring the inhibition zone directly by placing a rule on the bottom of the dish.)

Note: If necessary, the probable zone of growth inhibition may be tentatively estimated from eight to ten hours after addition of penicillin to the plates.

2. Record, from Table II, the probable concentrations of penicillin permitting and preventing growth in broth corresponding to the average diameter of the inhibition zones observed.

#### SUMMARY AND CONCLUSIONS

1. The basis of a technique for evaluating resistance of bacteria to penicillin by determining the zone of growth inhibition when a 4 mm. loopful of penicillin solution (10 or 5 Oxford units per cubic centimeter) in phosphate buffer is placed on the surface of nutrient or blood agar, seeded with a test organism, is developed in detail.

2. A table is presented which shows the relation of the zone of growth inhibition (produced by placing a 4 mm. loopful of a solution containing 10 or 5 Oxford units of penicillin per cubic centimeter on the surface of a seeded agar plate) to the probable concentrations of penicillin which will prevent or permit growth of staphylococci in nutrient broth.

3. The loop growth inhibition zone technique described for determination of bacterial resistance to penicillin is subject to less error, requires no special apparatus and considerably less media and equipment, and is not subject to the hazard of contamination associated with dilution techniques. The resistance can generally be tentatively determined within eight to ten hours after addition of penicillin to seeded plates.

4. When employing a penicillin solution containing 10 Oxford units per cubic centimeter with the technique described, growth inhibition zones of less than 10 mm. in diameter are considered to indicate that the test organisms are resistant; organisms producing growth inhibition zones of from 10 to 15 mm. in diameter are considered to be on the border line; and bacteria which show growth inhibition zones greater than 15 mm. in diameter are considered to be so susceptible as to warrant expectation of a satisfactory response to present methods of penicillin therapy.

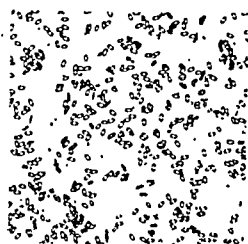
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## A RAPID METHOD FOR THE IDENTIFICATION OF CERTAIN SULFONAMIDE DRUGS

MARY C. DODSON, M.S., AND W. R. TODD, PH.D.  
PORTLAND, ORE.

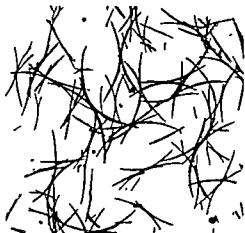
THE following method of identification of sulfonamide drugs is based on previous findings<sup>1, 2</sup> that certain of these compounds form crystalline cuprous complexes when heated in an alkaline copper sulfate solution containing a reducing sugar. The crystals are specific and reproducible for an individual



SULFANILAMIDE



SULFATHIAZOLE



DIMETHYL SULFADIAZINE



SULFADIAZINE



SULFAPYRIDINE



MONOMETHYL SULFADIAZINE

Fig. 1.—Cuprous-copper sulfonamide compounds ( $\times 100$ ).

From the Department of Biochemistry, University of Oregon Medical School.  
Received for publication, June 11, 1945.



drug and can be prepared in a few minutes. Identification appears to be more positive by this procedure than by the method of Sample recently reported.<sup>3</sup>

*Technique.*—Five cubic centimeters of Shaffer Hartman reagent,\* 5 c.c. of 100 mg. per cent glucose solution, and about 30 mg. of the sulfonamide drug are placed in a test tube 1 by 8 inches. After heating the mixture for five minutes in a boiling water bath, the crystals formed are examined microscopically. Small amounts of cuprous oxide may form with certain of the drugs, but this does not interfere with the crystal examination.

A description of the crystals is avoided since their preparation is simple, and any individual desiring to use this method of identification would undoubtedly prepare a series of the compounds to become familiar with their microscopic appearance. The structures vary greatly from drug to drug, but under similar conditions of alkalinity and concentration any one drug shows a remarkably constant crystal structure.

Sulfaguanidine and N<sup>4</sup> substituted sulfonamides do not react with cuprous copper under these conditions; consequently their identification cannot be carried out by this method. Sulfanilamide, sulfathiazole, sulfapyridine, sulfadiazine, mono-methyl sulfadiazine (sulfamerazine) and di-methyl sulfadiazine (sulfamethazine) are readily and positively identified by the procedure outlined.

Attempts to apply the method to urine containing sulfonamide drugs have been unsuccessful.

#### SUMMARY

A rapid method for the positive identification of sulfanilamide, sulfathiazole, sulfapyridine, sulfadiazine, sulfamerazine, and sulfamethazine is reported. This is done by microscopic examination of the crystalline cuprous complexes of these compounds.

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\*This is used for simplicity. Other solutions of comparable strength with respect to alkalinity and cupric ion are satisfactory.

## IMPROVEMENTS IN THE FLUORESCENT METHOD FOR DETECTING TUBERCLE BACILLI

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THE fluorescent method for detecting tubercle bacilli has several advantages. However, the published directions seem to give slides of varying quality. Bodies, other than tubercle bacilli, retain the auramine stain. These artefacts interfere with the identification of the tubercle bacilli.

The present method was devised to overcome these difficulties. This is accomplished by blending the sputum with a neutral medium. The blending

tends to separate the bacilli from the artefacts and make their location easy. A good blending agent is a water solution of methyl cellulose. This is sold under the trade name Methocel.\* The sales literature describes the properties of this material. The various types of methocel have different thickening powers. The highest viscosity type 4,000 cps seems to be the most useful for the present purpose.

The quality of the slides seems to be improved by treating the sputum with a water solution of pancreatic enzymes. The addition of dried bile to the enzyme solution may also be helpful in reducing the artefacts. The enzyme may act to reduce the artefacts because the latter are fatty substances that react with lipase, or the enzyme may alter the structure of the sputum so that better blending results. Good slides can be prepared without the enzyme treatment, and users may decide to simplify the method by its omission.

Complete directions will be given, which should allow ready duplication of the results.

#### REAGENTS

*Enzyme Solution.*—Mix 10 c.c. water, 0.5 Gm. of pancreatin (U. S. P.)† and 0.2 Gm. dried ox bile.‡ Allow to stand fifteen minutes and filter with No. 2 Whatman paper.

*Blending Solution.*—Mix 25 c.c. water and 0.5 Gm. of methocel type 4,000 cps. Boil gently for fifteen minutes and store in a refrigerator until a clear solution is obtained.

*Auramine Stain.*—Dissolve 0.1 Gm. of auramine 0§ in 10 c.c. ethyl alcohol, then add 90 c.c. water.

*Destaining Solution.*—Add 2 c.c. concentrated HCl to 98 c.c. water.

#### PROCEDURE

*Step 1.*—Select a small sample of sputum, add an equal volume of enzyme solution, and allow to stand thirty minutes.

*Step 2.*—Add an estimated 5 volumes of blending solution to the treated sputum and stir vigorously until a smooth mixture is obtained. Spread on the slide in a film of medium thickness, covering the entire slide except for one end. Air bubbles trapped in the smear disappear in the processing. Proceed to step 3 without drying.

*Step 3.*—Immerse the slide in auramine stain for five minutes.

*Step 4.*—It is very important to remove all excess stain. The following method should be carefully followed, unless a better one can be devised.

(a) Immerse the stained slide for five minutes in water heated to about 40° C.

(b) Remove the slide, thoroughly stir the material on it with a glass rod, and allow the slide to dry partially for five minutes.

This washing and stirring is repeated until the slide is colorless, except for a slight yellow haze. The slides usually clear on the sixth washing. This step ends with a final stirring and drying for five minutes.

*Step 5.*—Immerse the slide for exactly one minute in the destaining solution. Remove the slide, stir the material, and allow the slide to dry for five minutes; then rinse the slide in cold water, dry, and examine.

\*Dow Chemical Co., Midland, Mich.

†Eli Lilly and Company, Indianapolis, Ind.

‡Difco Laboratories, Inc., Detroit, Mich.

§National Aniline Division, Allied Chemical & Dye Corporation, New York, N. Y.

The usual method specifies 3 per cent phenol in the auramine stain, although Richards and co-workers<sup>1</sup> have obtained satisfactory results with 2 per cent phenol. The presence of these amounts of phenol modifies the methocel and causes partial disintegration of the smear. A phenol content of 1 per cent can be tolerated, but it seems better to omit it entirely. A considerable number of slides have been stained with stain containing no phenol and also 1 per cent phenol. The results seem to be satisfactory.

This general technique might be adapted to the identification of other organisms by fluorescent microscopy. Pringsheim and Vogel<sup>2</sup> list several "fluorochroms" which might be used as staining agents.

#### EXAMINATION OF THE SLIDES

Directions for converting an ordinary microscope for fluorescent work have already been published.<sup>3</sup> The CH4 mercury lamp works well, and a less efficient light source probably will not be satisfactory.

Slides should first be prepared from several positive sputa to show the various sizes and groups of tubercle bacilli. The bacilli can be located with the usual N. A. .66 objective and identified under oil immersion, using clear mineral oil. They appear very well with the usual N. A. 1.25 objective.

In case large numbers of slides must be examined, other objectives can be tried which sacrifice some resolution for convenience of use. The following objective was tested and seems to have advantages:  $40 \times 4.3$  mm., N. A. 1.00 fluorite oil immersion.\*

The magnification is rather low, but the good resolution, depth of focus, and area of field are attractive features. Dry objectives with N. A. .85 or .95 may also be adequate.

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\*Bausch & Lomb Optical Co., Rochester, N. Y.

## A SIMPLE COUNTER-RECORDER OF DROPS OR OTHER EVENTS

CLAUDE V. WINDER, Sc.D., AND VERNON A. MOORE  
DETROIT, MICH.

**M**ANY drop recorders have been described. Emphasis has usually been on modifications at the dropping end, with an ordinary signal magnet used for kymographic recording. To minimize the labor of counting recorded drops or other events, various adaptations of Fleisch's Ordinatschreiber have been used, but such special equipment as the latter is not readily available in many laboratories. In seeking a simple, easily assembled adding recorder, we resorted to the device illustrated in Fig. 1. It has been found most convenient and serviceable during about two years of use.\*

From the Research Laboratories, Parke, Davis & Co.

Received for publication, Aug. 6, 1945.

\*We are indebted to Mr. R. W. Thomas for the major part of this trial.

As we have used it, the recorder replaces the ordinary signal magnet. It is operated by the output of a 120 volt D.C. electronic relay activated by electrolytic closure of the input circuit through a drop falling between suitable platinum electrodes. The basic unit of the recorder is a commercially

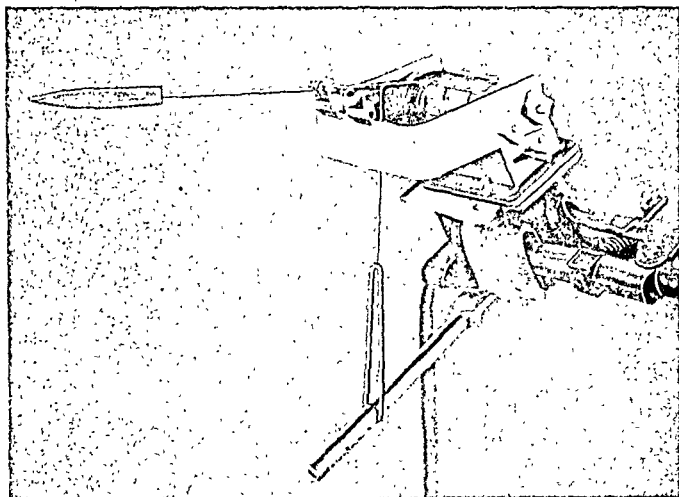


Fig. 1.—The recorder, mounted in an ordinary burette clamp, in operating position. Ten-event lever in place, with fulcrum provided for the hundred-event lever.

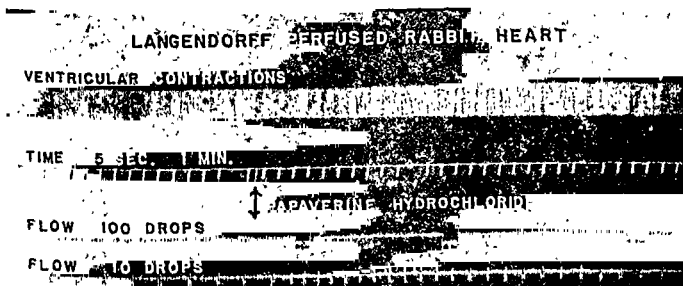


Fig. 2.—Illustration of simultaneous ten- and hundred-event recordings (last and next to last records). Note individual and tenth event signals in the one and individual and hundredth event signals in the other.

available electromagnetic counter\* built for 120 volts D.C.† A U frame is screwed to the ends of the counter case for mounting one, two, or three compact heart-lever bearings. Steel wire recording levers are shaped to operate at these fulcra to record vertically with respect to one another and to ride at

\*Gorrell and Gorrell High Speed Electric Counter, by Gorrell and Gorrell, Chicago Heights, Illinois.

†Other current capacities in relay and counter could be used, according to the current source most conveniently available.

The contributions on the vitamins seem well suited to human consumption although written in most instances by authorities in agricultural colleges and laboratories of biochemistry. This reviewer finds it rather stimulating to be told what the "clinical possibilities" of a B vitamin may be long before the vitamin has been tried clinically.

The purpose of the book as set forth in the foreword might have been better accomplished by some editing to bring the nutritionists and the dietotherapists into closer agreement. For instance, the foreword written in 1942 in part by a committee of the National Research Council states that the physician may do with a "biomicroscope" in examination of patients what the pathologist has for the methods of pathology by his microscope. However, in 1945 there seems to be no contributing author who even mentions the biomicroscope. Likewise, the foreword's criticisms of customary corrective diets used in gastroenterology, as in peptic ulcer, seem unheeded in the section dealing with ulcer. Perhaps this discrepancy is a fault of medical practice rather than one of editing a book.

On the whole, the nutritional theorists have not run away with this book. It is a good text on dietotherapy, even though it frequently calls for "easily digested" food without ever explaining in a most thorough chapter on digestion just what "easy digestion" is.

This shows that dietotherapy is still a useful art, even if outstripped by nutritionists. The book is, in the opinion of the reviewer, as good a junction of theoretical nutrition with practical dietetics as can now be made.

M. A. BLANKENHORN, M.D.

**Fundamentals of Pharmacology.** By *Clinton H. Thienes*, M.D., Ph.D., Professor of Pharmacology, University of Southern California School of Medicine, Attending Pathologist (Toxicology), Los Angeles County Hospital. Paul B. Hoeber, Inc., New York. Price \$5.75. Cloth with 497 pages.

This volume is one of a series of texts which are being edited by Dr. Fred C. Zapffe as secretary of the Association of American Medical Colleges. The object in writing these books is to present authoritatively the basic foundation and to omit nonessentials in the subjects covered. The aim is to create books the student will have time to read in spite of the great pressure which is put upon him from all directions. This is a splendid idea and the need for progress in this direction is long since overdue.

The present volume covers the field of pharmacology in twelve sections as follows: (1) Stimulants of the Central Nervous System; (2) Depressants of the Central Nervous System; (3) Drugs Acting on the Peripheral Nervous System; (4) Drugs Acting on Muscles; (5) Diuretics; (6) Antiparasitic Drugs; (7) Hormones, Special Minerals, and Tissue Extracts; (8) Vitamines; (9) Drugs Used Because of Local Action on Body Surfaces; (10) Chemical Diagnostic Agents; (11) Actions of Drugs on Cells; (12) Pharmacy and Prescription Writing.

This is an admirable small text. In most instances it presents more of the pharmacology of any given drug than the average medical student will be able to learn in the time, and with the opportunities, at his disposal. No attempt is made to cover in detail a vast array of drugs, but practically all of those in extensive use at present are discussed, including a series of the sulfa drugs, demerol, and penicillin. The subject of Prescription Writing is covered in eighteen pages, English being used in preference to Latin. A useful bibliography follows each general section throughout the book.

In general, this will be a very helpful book for medical students, especially for review purposes.

D. E. J.

# THE ANTITUBERCULAR ACTIVITY OF ASPERGILLIC ACID AND ITS PROBABLE MODE OF ACTION

ANDRES GOTH, M.D.

DALLAS, TEXAS

IT HAS been reported in the recent literature<sup>1-3</sup> that various *Aspergillus* species produce inhibitory substances against *mycobacterium tuberculosis*. Because of the obvious significance of these observations, a study was undertaken in order to determine the possible inhibitory effect of the various antibiotics produced by the mold *aspergillus flavus* against the tubercle bacillus.

It has been shown during the last five years that molds belonging to the species *A. flavus* produce several antibiotic substances. White and Hill<sup>4</sup> showed that a moderately toxic antibiotic was produced by this mold which he named aspergillie acid. Bush and Goth<sup>5</sup> described a penicillin-like substance produced by an *A. flavus* which was named flavicin. Aspergillie acid inhibited a great variety of bacteria, including both gram-positive and gram-negative species. Its activity against *Myco. tuberculosis* has not been described.

On testing extracts obtained from *A. flavus* cultures against various strains of *Myco. tuberculosis* it was found that while flavicin had no inhibitory effect on this organism, aspergillie acid showed marked inhibitory properties. In addition to this finding, the experiments in which this inhibitory effect was studied threw some light on the probable mechanism of action of aspergillie acid.

## MATERIAL AND METHODS

Aspergillie acid was obtained from various sources. Partially purified material was prepared in the following manner: a strain of *A. flavus* was grown on a medium containing 2 per cent bacto-tryptone,\* 2 per cent dextrose† (U. S. P.), and 0.5 per cent NaCl (C. P.). Eight- to ten-day-old cultures were extracted with butyl acetate at pH 4 in a spray extraction column.<sup>6</sup> The active material was recovered from the solvent by shaking it with 0.1 M NaHCO<sub>3</sub> solution. The material was further purified by adsorbing the toxic impurities on charcoal (Darco G 60). Results obtained with this partially purified material were confirmed by using purified aspergillie acid.†

Two strains of *Myco. tuberculosis* were used in this study. One was the H 37 strain obtained from the American Type Culture Collection. The other was a fast-growing human strain.†

The media for growing the tubercle bacillus were Long's synthetic medium, with and without ferrie ammonium citrate (5 mg. per 100 c.c.), and bacto nutrient broth.\* On the latter medium only the fast-growing tubercle

From the Department of Physiology and Pharmacology, Southwestern Medical College. Funds for carrying on this work were kindly given by Eli Lilly and Company.

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\*Difco Laboratories, Inc., Detroit, Mich.

†Obtained from the Squibb Research Institute through the courtesy of Drs. G. Rake and C. M. McKee.

‡Obtained from Dr. R. C. Avery of Vanderbilt University.

bacillus multiplied readily. All media were distributed in 5 c.c. portions. Liquid media were used for the fast-growing strain: agar was added for growing the H 37 strain. Aspergillie acid was added to the media as the sodium salt.

### RESULTS

When purified aspergillie acid was tested against a fast-growing strain of *Myco. tuberculosis* in broth, complete inhibition was observed up to a 1:80,000 dilution. When the same experiment was carried out in Long's synthetic medium (containing 5 mg. per 100 c.c. ferric ammonium citrate) no inhibition was observed even at 1:20,000.

Since it seemed likely that some substance in Long's medium interfered with the antitubercular activity of aspergillie acid, each one of the constituents of this medium was examined systematically for its ability to interfere with the activity of the antibiotic. When ferric ammonium citrate was excluded from the medium, the inhibitory effect of aspergillie acid was the same as in broth. Since ferric chloride had the same effect as ferric ammonium citrate, it was concluded that the ferric ion is responsible for the inactivation of aspergillie acid.

The results of a typical experiment are represented in Table I. Aspergillie acid was markedly inhibitory against the tubercle bacillus in media to which no iron was added, whereas this antibacterial effect disappeared following the addition of ferric ammonium citrate (5 mg. per 100 c.c.) to the medium. The results were evident in three days and remained unchanged for two weeks.

TABLE I.—THE INHIBITORY ACTIVITY OF PURIFIED ASPERGILLIC ACID ON A FAST-GROWING HUMAN STRAIN OF *MYCO. TUBERCULOSIS*

CONCENTRATION OF ASPERGILLIC ACID	AMOUNT OF GROWTH		
	NUTRIENT BROTH	LONG'S MEDIUM WITHOUT IRON	LONG'S MEDIUM WITH IRON
1:20,000	0	0	+++
1:40,000	0	0	+++
1:80,000	0	0	+++
0	+++	++	+++

While carrying out these experiments the observation was made that when aspergillie acid was added to the medium containing ferric ammonium citrate or ferric chloride, a fine red precipitate appeared within two to three minutes in the medium. This red substance, which was evidently not inhibitory, was actually taken up by the tubercle bacillus as evidenced by the bright red appearance of the colonies.

When these experiments were repeated using the slow-growing H 37 strain of *Myco. tuberculosis*, the findings were essentially the same. Aspergillie acid inhibited the growth of this organism in a medium to which no iron was added. In the presence of ferric ammonium citrate, the colonies of the H 37 strain showed an accumulation of the red substance which originated from the interaction of aspergillie acid and ferric ammonium citrate. However, these red colonies, as well as the controls to which no aspergillie acid had been added, failed to grow.

It was of interest to determine whether or not ferric ion would interfere with the antibiotic activity of aspergillie acid against organisms other than the tubercle bacillus. On testing this possibility the results were the same as

with the fast-growing strain of *Myco. tuberculosis*. Purified aspergillie acid inhibited the growth of a *Staphylococcus aureus* when added to broth in the amount of 1:80,000. Following the addition of ferric ammonium citrate (5 mg. per 100 c.c.) the inhibitory activity was not present at 1:20,000.

The growth of both strains of *Myco. tuberculosis* was much heavier in media to which iron was added than in media which had only traces of iron. This observation is in agreement with the findings of Schmidt,<sup>7</sup> according to whom the addition of iron to culture media stimulates the growth of *Myco. tuberculosis*.

On further study of the nature of the interaction between ferric ions and aspergillie acid, the following experiment was carried out. To 2 mg. of ferric ammonium citrate 5 c.c. of a partially purified aspergillie acid solution was added, representing 750 staphylococcus units, which correspond to approximately 10 mg. of purified material. A red precipitate appeared which was insoluble on acidification with H Cl and was readily soluble when the solution was made alkaline by the addition of 0.1 M NaOH. When the precipitate was filtered from the acidified solution, the filtrate failed to give the Prussian blue test for the presence of ferric ions. When the precipitate was ashed and subsequently dissolved in water, it gave a positive test with potassium ferrocyanide. From these qualitative tests it appeared clear that ferric ions were precipitated by aspergillie acid. Since the antibiotic activity changed simultaneously with the appearance of the precipitate, it is reasonable to assume that the red substance which appears whenever ferric ions are added to aspergillie acid represents an aspergillie acid-iron complex.

Since iron was found to interfere with the antibiotic activity of aspergillie acid, it was of importance to find out whether or not blood which contains mostly ferrous iron bound in a complex organic linkage, would interfere with the activity. To test this possibility, aspergillie acid was added in the amount of 1 mg. per cubic centimeter to defibrinated rabbit blood, which subsequently was tested for antibacterial activity. It was found that aspergillie acid added to blood still inhibited the growth of *Staph. aureus* and the fast-growing strain of *Myco. tuberculosis* up to 1:40,000, the highest dilution tested.

#### DISCUSSION

From the experiments reported, the following facts appear:

1. Ferric ion stimulates the growth of *Myco. tuberculosis*.
2. Aspergillie acid inhibits the growth of *Myco. tuberculosis* in media to which no ferric ions are added.
3. Ferric ions interfere with the antibiotic activity of aspergillie acid.
4. Aspergillie acid precipitates ferric ions.

The most likely explanation of these findings can be given in the following hypothesis: iron is essential for the growth of *Myco. tuberculosis*; aspergillie acid inhibits the growth by interfering with the utilization of iron or perhaps also by interfering with the functioning of iron-containing enzyme systems.

The inhibitory activity of aspergillie acid against the tubercle bacillus is sufficiently high to encourage chemotherapeutic trial in experimental animals. Such studies are being carried out at the present time in guinea pigs, and the outcome of these studies will be reported later. If these experiments indicate any in vivo antitubercular activity, clinical trials will be carried out; also, the



effect of various metals on the activity and toxicity of aspergillie acid is being investigated.

### CONCLUSION

Aspergillie acid in low concentrations inhibits the growth of human strains of *Myco. tuberculosis*. Suggestive evidence is presented which indicates that the mode of action of aspergillie acid can be explained on the basis of interference with the utilization of iron by the tubercle bacillus.

The author wishes to acknowledge the technical assistance of Jane C. Neff.

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### SEROLOGIC REACTIONS IN PRIMARY ATYPICAL PNEUMONIA

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PRIMARY atypical or viroid<sup>1</sup> pneumonia was the form of pneumonitis encountered most frequently in World War II. The clinical picture has become familiar as a result of a great many reports, and extensive studies have been made during the past few years into its epidemiologic and laboratory aspects.<sup>2</sup> Although the viral nature of the etiologic agent is strongly suggested by the transmission of the disease to human volunteers with bacteria-free filtrates,<sup>3</sup> the properties of the agent or agents are yet to be described. It is clear that the majority of instances of the syndrome are not caused by the viruses described by Weir and Horsfall<sup>4, 5</sup> and by Eaton and associates<sup>6</sup> as producing pneumonitis in the mongoose and cotton rat, respectively. Neither are they due to the influenza nor any of the other familiar viruses. However, a number of characteristic changes have been observed to develop in the sera of patients during convalescence. These have been reflected in the cold agglutinin test,<sup>7, 8</sup> indifferent streptococcus agglutination test,<sup>9</sup> elementary body type virus complement fixation test,<sup>10-12</sup> and in the serologic tests for syphilis.<sup>13-15</sup> Changes have also been reported to occur in the Weil-Felix test.<sup>16</sup>

The present communication is a report of the results of subjecting two or more serial specimens of serum from 100 patients, sixty-eight of whom were

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clinically diagnosed as having primary atypical pneumonia, to one or more of a "battery" of these tests. Studies were made of the cold agglutinin titer (CA), indifferent streptococcus agglutination (SA), elementary body type virus complement fixation (EB), and Kahn flocculation and Kolmer complement fixation tests for syphilis. In addition, a report is made of the results obtained when a group of seventy-three consecutively received single specimens of serum submitted for cold agglutinin testing were also examined in the sheep cell heterophil and Weil-Felix tests.

#### MATERIALS AND METHODS

*Sera.*—Sera were obtained from patients at army hospitals scattered throughout the southern half of California, Utah, Nevada, and Arizona. They were all originally submitted for cold agglutinin or influenza agglutination-inhibition antibody examinations. For the purposes of the present study, only those serial specimens of serum from patients with illnesses which had been diagnosed as "primary atypical pneumonia," "virus pneumonia," or "virus bronchitis" were included in the "primary atypical pneumonia" group. All others, which included those from patients with diagnoses of "bronchopneumonia," "pneumonitis," "lobar pneumonia," "questionable influenza," and "nasopharyngitis," were put in the "control" group. This rigid division of cases seemed necessary since in almost every instance contact between this laboratory and the actual patient was limited to correspondence with the patient's medical officer.

The hospital laboratories were requested to separate the serum from freshly clotted blood. If the blood had been stored in the cold, they were asked to bring it to body temperature before making this separation. In most instances the serum was sent to this laboratory through the regular mail with sulfanilamide as a preservative.<sup>17</sup> When this had not been done, the serum on arrival was transferred to such a "sulfa vial" and stored in the refrigerator until tested. The cold agglutinin (CA), elementary body virus complement fixation (EB), and serologic tests for syphilis were usually done shortly after the specimens were received. The indifferent streptococcus (SA) agglutination test on about one-half of the specimens was done from two to twelve months after receipt. This seemed justifiable since it has been demonstrated that the SA is well preserved for long periods at refrigerator temperature.<sup>9</sup>

*Cold Agglutinin Test (CA).*—The technique described by Horstmann and Tatlock<sup>7</sup> was followed. However, the sera were inactivated at 56° C. for fifteen minutes prior to dilution to destroy any complement which might be present. The tests were read after storage overnight in the refrigerator at approximately 0 to 4° C. and again after warming in the water bath at 37° C. for fifteen minutes. A known positive serum was included in every run as a check on the sensitivity of the red cells being used. Variation in titer of more than one dilution in this control disqualified the run. The tests were read very closely and the least perceptible agglutination, when compared with the cell control, was called positive. Agglutination in a dilution of 1 to 16 or greater was considered significant.

*Indifferent Streptococcus Agglutination Test (SA).*—The method described by Thomas and associates<sup>9</sup> was adhered to in exact detail. A culture of the streptococcus 344 was obtained from Dr. M. D. Eaton. When results were read, only tubes in which there was definite agglutination and clear fluid were called positive. Titers of 1 to 10 or greater were considered significant.

*Elementary Body Type Virus Complement Fixation Test (EB).*—“Ly-granum CF”<sup>\*</sup> was used as antigen and the technique previously described by one of us was followed.<sup>12</sup> The lowest dilution of serum tested was 1 to 10. No single titer, only a change in titer, was considered significant.

*Kahn Flocculation Test for Syphilis.*—The standard Kahn procedure<sup>18</sup> was followed, but only a single reading, after fifteen minutes, was made. The antigen was furnished by the Army Medical School. Readings which gave a total of from 7 to 12 plus were called positive, those with 2 to 6, doubtful, and less than 2, negative.

*Kolmer Complement Fixation Test for Syphilis.*—The modified micro-Kolmer test, previously described,<sup>12</sup> was used. The antigen was obtained from the Army Medical School. With the patient's serum diluted 1 to 5, 3 or 4 plus fixation was called positive. If any other fixation was present, the test was reported as doubtful.

*Heterophil Test.*—The shaking and spinning technique described by Crawford and Hertert<sup>19</sup> was followed. Defibrinated sheep cells, never more than three days old, were used. A titer of 1 to 40 or greater was considered significant.

*Weil-Felix Test.*—The method of Crawford and Hertert<sup>19</sup> was employed. Antigens were prepared from *Proteus* OX-2, OX-19, OX-K strains. A titer of 1 to 80 or greater was considered significant.

In the interpretation of these tests only a rise or fall in significant titers was considered positive. If a significant titer was maintained unchanged in all specimens, or if the peak antibody titer was only “doubtfully positive,” it was interpreted as a questionable reaction.

## RESULTS

In Table I is presented a summary of the results when each of the four tests in the “battery” was applied to serial specimens of serum from patients with primary atypical pneumonia. Many of these specimens were inadequate for more than one of the serologic tests for syphilis. Therefore, in the table, the results of both Kahn and Kolmer are grouped together under the simple heading Serologic Test for Syphilis (STS). A positive or doubtfully positive transitory reaction in either of these tests is accordingly indicated.

TABLE I. INCIDENCE OF REACTIONS IN GROUP OF PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA

TEST	TOTAL NUMBER OF PATIENTS TESTED	NUMBER OF TESTS			POSITIVE IN EACH GROUP (%)
		POSITIVE	DOUBTFUL	NEGATIVE	
Cold agglutinin (CA)	68	43	7	18	63
Indifferent streptococcus agglutinin (SA)	36	17	8	11	47
Elementary body complement fixation (EB)	45	12	4	29	26
Serologic for syphilis (STS)	36	6	6	24	17

Sera from sixty-eight patients were studied by us with the CA test. Of these, forty-three (63 per cent) gave a positive reaction. This result may be compared with the forty-three of ninety-three patients (46 per cent) with primary atypical pneumonia studied by the Commission on Acute Respiratory Diseases of the United States Army<sup>8</sup> who had maximum titers of 1 to 16 or

\*E. R. Squibb & Sons, New Brunswick, N. J.

above, and the sixty of seventy-four patients (81 per cent) reported by Meikeljohn<sup>20</sup> who had titers of at least 1 to 20. These differences may reflect the geographic sources of patients and criteria for selection of cases. The patients reported by the Commission were all soldiers admitted to the Station Hospital at Fort Bragg, N. C., and seen by at least one member of the Commission. Those in Meikeljohn's study were mostly civilians and college students from the San Francisco Bay region. They were all selected by him. Our patients were drawn from Army posts in various parts of California, Utah, Nevada, and Arizona, where the original diagnoses were made by many different physicians. However, all reports agree that in a considerable percentage of patients with this syndrome, cold agglutinins do develop during convalescence.

There were thirty-six sets of sera which were also examined by us in the SA test. Of these, seventeen (47 per cent) were positive. Although this is a small series of patients, the results are strikingly similar to those reported by other investigators. Thomas and associates<sup>9</sup> found that fifty-five of 101 (55 per cent) and Meikeljohn and Hanford<sup>21</sup> reported that seventy-three of 156 patients (47 per cent) had titers of at least 1 to 10.

The sera of only twelve of the forty-five patients (26 per cent) we studied with the EB test were positive. The use of the EB test in the study of primary atypical pneumonia has already been discussed by one of us.<sup>12</sup> It probably segregates those instances of the syndrome caused by viruses of the ornithosis-psittacosis group. Since the test does not differentiate between the members of this group, its interpretation should not be carried beyond this general statement. Our "Lygranum CF" antigen crosses serologically with the psittacosis virus, and it is therefore of interest that Rein and Elsberg<sup>14</sup> reported that nineteen of a group of thirty-six patients with primary atypical pneumonia (53 per cent) had positive complement fixation tests for psittacosis.

Thirty-six of our patients had consecutive serologic tests for syphilis performed. Of these, six (17 per cent) had transitory, definitely positive serologic tests for syphilis develop during convalescence. An additional six showed transitory doubtfully positive reactions. Therefore, a total of twelve (33 per cent) in this small series of patients developed some change in the serum globulins which could be detected with the serologic test for syphilis. It should be noted that all of these were, in reality, "false" positive reactions. In 1941 Hegglin<sup>13</sup> reported in the Swiss literature nineteen cases of an atypical pneumonia with repeated strongly positive transitory serologic tests for syphilis and suggested that this was a new syndrome. Recently at the Army Medical School in Washington, D. C., Rein and Elsberg<sup>14</sup> subjected serial specimens of serum from thirty patients with atypical pneumonia to a battery of six serologic tests for syphilis and found that seventeen (24 per cent) had transitory positive reactions in two or more of these tests.

In Table II is presented a summary of the results when the CA, SA, and STS were applied to the "control" sets of sera.

TABLE II. INCIDENCE OF REACTIONS IN GROUP OF CONTROL PATIENTS WITH VARIOUS RESPIRATORY DISEASES

TEST	TOTAL NUMBER OF PATIENTS TESTED	NUMBER OF TESTS			POSITIVE IN EACH GROUP (%)
		POSITIVE	DOUBTFUL	NEGATIVE	
Cold agglutinin (CA)	32	6	0	26	19
Indifferent streptococcus agglutinin (SA)	25	3	3	19	12
Serologic for syphilis (STS)	22	2	6	14	9

The fact that six of thirty-two (19 per cent) of these individuals gave a positive CA test seems strikingly high. However, a review of the clinical diagnoses in these positive cases suggests that some of these may actually have been instances of primary atypical pneumonia which were incorrectly classified due to our arbitrary division of cases. Two of the patients were diagnosed as having "br  nchopneumonia," one "pneumonitis," one "lobar pneumonia," one "bronchiectasis," and the last "nasopharyngitis."

Three of the twenty-five patients tested with the SA reaction gave positive results. Diagnosis in one of these was "questionable influenza" and in the other two, "nasopharyngitis."

There were two transitory positive STS tests among the twenty-two sets of sera examined. Both of these were in patients with "nasopharyngitis"; in addition, one had a positive CA and negative SA, while the other had a positive SA and negative CA test.

Although our "control" group is small, it does tend to indicate that a positive CA or SA test in a patient with respiratory disease is strong presumptive evidence in favor of a diagnosis of "primary atypical pneumonia."

Since the CA test was found to have the highest incidence of positive reactions among patients with primary atypical pneumonia, an attempt was made to correlate the results in the SA, EB, and STS tests with those in the CA test. This is shown in Table III. In our limited series it appears that the majority of positive SA tests occurred in individuals who had developed positive CA reactions. This was also Meikeljohn and Hanford's<sup>21</sup> experience. However, most of our positive EB tests were in patients who did not have an increase in cold agglutinins. "False" positive serologic tests for syphilis were found to be unrelated to the distribution of positive CA tests. In Table III is also indicated that the CA and SA tests reflect different antibodies. There were five patients who had positive CA reactions and negative SA tests and four who had positive SA reactions and negative CA tests. Evidence that the EB and STS are not related to each other has been presented elsewhere.<sup>12</sup> These four tests would therefore seem to reflect at least four distinct antibodies which might arise in an individual as a consequence of his having an attack of atypical pneumonia.

TABLE III. DISTRIBUTION OF OTHER REACTIONS IN RELATION TO REACTIVITY IN COLD AGGLUTININ TEST IN PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA

COLD AGGLUTININ	REACTIONS IN OTHER TESTS	INDIFFERENT STREPTOCOCCUS AGGLUTININ (SA)	ELEMENTARY BODY COMPLE- MENT FIXATION (EB)	SEROLOGIC TESTS FOR SYPHILIS (STS)
	+	11	4	3
Positive	?	4	3	3
	-	5	20	14
	+	2	2	0
Doubtful	?	0	0	1
	-	2	3	3
	+	4	6	3
Negative	?	4	1	2
	-	4	6	7
Total patients in each test		36	45	36

When the results of these tests are summarized for each patient, as is done in Table IV, patterns of serologic reactivity or "profiles" emerge. However, among twenty patients with primary atypical pneumonia whose serial specimens of serum were examined with this "battery" of tests, fifteen different profiles appeared!

TABLE IV. REPRESENTATIVE REACTIONS OF PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA

PATIENTS	SPEC. NO.	DAYS AFTER ONSET	COLD AGGLUTININ (CA)	INDIFFERENT STREPTOCOCCUS AGGLUTININ (SA)	ELEMENTARY BODY COMPLEMENT FIXATION (EB)	SEROLOGIC TESTS FOR SYPHILIS STS	
						KAHN	KOLMER
1. Primary atypical pneumonia	1424	10	16,384				N
X-ray, +	1454	31	256	<10	<10	N	
W.B.C., 6,000	1464	39	<16	<10	<10	N	N
Profile			+	-	-		-
2. Primary atypical pneumonia	5096	24 days before onset	<16	<10			
X-ray, +	5106	7	256	40	<10		N
W.B.C., 5,800	5112	35	256	40	<10	N	N
Profile			+	+	-		-
3. Primary atypical pneumonia	1769	15	256	40	<10	01±	+1
X-ray, +	1781	25	1024	80	<10	N	N
W.B.C., 3,700	1786	43	1024	20	<10	N	N
Profile			+	+	-		?
4. Primary atypical pneumonia	1691	13	16	80			+4
X-ray, +	1698	23	<16	160	<10		+4
W.B.C., 5,600	1744	54	<16	40	<10	N	N
Profile			+	+	-		+
5. Primary virus bronchitis	1358	5	<16	10	10	N	N
X-ray, -	1379	16	64		20		+4
W.B.C., 8,400	1401	36	<16	80	<10	N	N
Profile			+	+	+		+
6. Primary atypical pneumonia	733	18	<16	<10	80	N	N
X-ray, +	804	25	<16	18	80		N
W.B.C., 7,500	964	48	<16	<10	10	N	+4
Profile			-	+	+		+
7. Primary atypical pneumonia	1346	9	<16	<10	<10	N	+2
X-ray, +	1362	28	<16		20		N
W.B.C., 10,000	1380	41	<16	<10	10	N	N
Profile	1416	71	<16		<10		N
8. Primary atypical pneumonia	1774	13	<16	<10	<10	N	N
X-ray, +	1783	27	<16	10	<10	N	N
W.B.C., 9,750	1796	41	<16	10	<10	N	N
Profile			-	+	-		-

A review of our data for the time of appearance of these various antibodies revealed a general conformity to what has been previously reported by others.<sup>7, 14, 21</sup> Although in a few instances the CA and SA were apparent within the first week of illness, the peak of CA usually came between the tenth and twenty-fifth days, while that of SA usually did not appear until after the third week. The EB antibody appeared during the second and third weeks when it also reached its peak. The most frequent time for appearance of reagin as measured by the STS was the second week, although in an occasional patient it was not detected until much later.

Early in our study, seventy-three consecutively received specimens of serum from patients with the diagnosis of primary atypical pneumonia were tested with the CA, heterophil, and Weil-Felix reactions. This was prompted by a report that Cheney and Gardner<sup>16</sup> had found that seven of eight of their patients with atypical pneumonia who were tested had positive Weil-Felix tests. The heterophil test was included, since the clinical picture of infectious mononucleosis, in which there is a high incidence of positive reactions, may resemble atypical pneumonia. In our group of seventy-three specimens there were twenty with significant CA titers. Two had heterophil levels of 1 to 40, and only one had a significant Weil-Felix titer.

## DISCUSSION

The development of so many different combinations of the antibodies which are indicated by the CA, SA, EB, and STS in our group of clinically similar cases of pneumonitis suggests again that primary atypical pneumonia is a syndrome which can be produced by several different agents. As indicated by the results of the EB tests, a number of our cases were probably caused by some member of the ornithosis group of viruses. We also know that occasionally the influenza virus, the rickettsia of Q fever,<sup>22, 23</sup> and even the fungus *Coccidioides immitis*<sup>24</sup> may simulate this picture. This is not unlike the familiar difficulty of attempting to recognize clinically the significant differences between the various encephalitides and forms of dysentery.

It would be convenient to think that all the patients who developed positive CA tests represented infections with a single, as yet unknown, etiologic entity. In support of this concept are the facts that it was from this group that Eaton recovered a virus which produced pneumonitis in cotton rats<sup>6</sup> and from which the transmission of the human disease with bacteria-free filtrates was demonstrated.<sup>3</sup> However, it leaves unexplained the appearance of cold agglutinins in an occasional patient with a positive EB test, the not invariable association of cold agglutinins with the development of indifferent streptococcus agglutinins, and the relatively high incidence of false positive STS<sup>15</sup> among patients with this syndrome. It has been suggested that primary atypical pneumonia might be caused by the synergistic action of several agents; for example, a virus and bacterium, as in swine influenza. Although we prefer not to think of a multiplicity of agents as the basis of a single illness, it may offer the only logical explanation for the multiplicity of serologic reactions. On the other hand, Landsteiner<sup>25</sup> has pointed out that "complex materials can give rise to as many antibodies as there are antigenic components." Consequently, a single very complex agent might conceivably be responsible for the more frequently recognized atypical pneumonias and the variety of serologic profiles might represent but another example of individual variation in immunity. Fascinating deductions as to the antigenic composition of this agent follow when the nature of the antigens we used for detecting these antibodies are considered. Thus it has been shown that the active principle in the indifferent streptococcus is a polysaccharide,<sup>26</sup> in the STS it is a cardiolipid,<sup>27</sup> and in the CA test it is contained in the stroma of the human erythrocyte.<sup>28</sup>

Intriguing as this may be, at the present state of our knowledge we should merely note that from 40 to 80 per cent of patients with the syndrome of primary atypical pneumonia develop an increase to significant levels of cold agglutinins; that those who develop CA are usually the ones in whom SA appear, and that the majority of individuals who have positive EB tests do not have positive CA reactions. The relative frequency of so-called "false positive" serologic tests for syphilis in this syndrome is also worthy of note.

## SUMMARY

1. Serial specimens of serum from sixty-eight patients with a clinical diagnosis of primary atypical pneumonia were examined in one or more of a "battery" of serologic tests. The tests employed were the cold agglutinin (CA), indifferent streptococcus agglutinin (SA), elementary body type virus complement fixation (EB), and the Kahn flocculation and Kolmer complement fixation tests for syphilis (STS).

3. It was demonstrated that a positive SA test is more likely to occur in a patient with a positive CA reaction, that the majority of individuals with positive EB tests do not have positive CA tests, and that there is no apparent relationship between the occurrence of any of these reactions and a transitory positive STS.

5. The frequency with which CA, SA, EB, and STS antibodies are found to develop in patients convalescing from primary atypical pneumonia suggests an immunologic association with the etiologic agent (or agents) of the disease. The many combinations of response which are measured by the tests reported here may be a reflection of individual variation or of several different agents producing the same syndrome. Until the etiologic agent for the majority of instances of primary atypical or viroid pneumonia is identified, these tests serve the practical purpose of more clearly defining the disease.

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## PHAGOCYTTIC ACTIVITY OF NEUTROPHILES IN ANEMIAS

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FOR years students of anemia have pondered over the physiologic mechanisms that enable the severely anemic patient with white cell counts of from 1,500 to 3,000 per cubic millimeter to avoid overwhelming infections. The question has also been raised as to how these patients are able to walk about and maintain a mild activity with red cell counts around a million and hemoglobin values of from 20 to 30 per cent. As yet there is no complete answer for either of these problems, but it appears that we have found at least a partial explanation for the anemic patient's defense against infections. In studying the phagocytic activity of neutrophiles in malnourished patients seen at the Nutrition Clinic of Hillman Hospital in Birmingham, we observed in one of our most poorly nourished patients, who also had a macrocytic hyperchromic anemia, a greatly increased phagocytosis. As more patients with anemia were tested we discovered that without exception patients with either macrocytic hyperchromic or microcytic hypochromic anemia had an increased white cell activity and that the increase in phagocytosis was roughly proportional to the severity of the anemia.

### METHODS

Phagocytic activity was measured on whole blood according to the method of Cottingham and Mills.<sup>1</sup> Briefly, this consists of diluting in a paraffined serologic tube 0.5 c.c. of venous blood with 0.5 c.c. heparinized† saline; 0.2 c.c.

University of Cincinnati Studies in Nutrition at the Hillman Hospital, Birmingham, Ala. From the Department of Internal Medicine, University of Cincinnati.

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†The heparin was very kindly supplied by Hoffman-La Roche, Inc., Nutley, N. J.

of a standardized suspension of *micrococcus candidus* is added to the tube, which is quickly gassed with a mixture of 95 per cent  $O_2$  and 5 per cent  $CO_2$ , stoppered, inverted twice, and agitated in a water bath at  $38^\circ C.$  for four minutes. Films are made immediately and then stained. A differential white cell count is made, and at the same time the number of bacteria in 100 neutrophils is counted and averaged. The figure obtained in this way is arbitrarily taken as the phagocytic activity of the blood. The minimum error of the method when control values from day to day are compared (see Table I, column 4) is approximately  $\pm 10$  per cent. The principal variable is the density of the bacterial suspension. This was standardized by means of an Evelyn colorimeter using a  $515 \mu\mu$  filter. Variations of from 2 to 3 scale divisions on the galvanometer produced changes in phagocytic counts of from 30 to 40 per cent. Another source of error appears as the average number of bacteria per neutrophil approaches 30. Since it is impossible to count accurately more than 30 bacteria within a cell, this is then taken as the largest number used in determining the phagocytic activity. If there is a relatively large number of leucocytes "filled" with bacteria, the average then becomes more inaccurate. Hence the error in such cases is probably at least from 20 to 30 per cent.

All of the results are based upon a comparison of the phagocytic activity of leucocytes of anemic patients with those of "normal" persons. The blood of one of us (L. J. B.) was used as the control, since he is free from any disease, consumes an excellent diet rich in proteins, minerals, and vitamins, and has always had normal blood during occasional tests over the past two years and frequent tests during the past five months. The white blood cell total has varied between 7,700 and 9,800, with a differential count always within normal limits of variation. The red cell total and hemoglobin have varied between 4.89 and 5.10 millions and 14.7 and 15.9 Gm., respectively. The phagocytic activity of this control blood checked within 10 to 15 per cent the phagocytic activity of the blood of other workers in the laboratory who are of both sexes and are in equally good health. One such worker (R. B. J.) was used as the "normal" on some days.

Each day an experimental test was made on the blood of anemic patients, "normal" blood was collected and treated identically. Variations due to changes in the density of the bacterial suspensions from one day to the next should give comparable results between the "experimental" and control blood samples. Thus in order to eliminate the day-to-day differences in phagocytic activity arising from the method itself, the value obtained for the blood of the anemic patient is divided by the value obtained for "normal" blood on the same day. This gives a calculated value which is designated as "per cent 'normal' phagocytosis."

#### RESULTS

The results from twenty-nine patients are shown in Table I. The phagocytic activity of the patient is given in column 3, while the "normal" phagocytic activity on the same date is shown in column 4. The five values marked with the asterisk were obtained with R. B. J. as the control. The per cent "normal" (numbers in column 3 divided by those in column 4  $\times 100$ ) shown in column 5 is more than 100 per cent in all cases. When the magnitude of these values is compared with the red cell counts (column 6) and hemoglobin level (column 7), it can be seen that in general the more severe the anemia, the higher the phagocytosis. The mean per cent "normal" phagocytosis in those

TABLE I

(1) CASE	(2) DATE	(3) PHAGOCYTTIC ACTIVITY	(4) "NORMAL," PHAGOCYTTIC ACTIVITY	(5) PER CENT "NORMAL,"	(6) R.R.C. (MILLIONS)	(7) H.G. (G.M.)	(8) W.B.C.	(9) PER CENT (P. M. N.)	(10) TYPE ANEMIA
1	5/3	22.38	9.93	225	1.74	7.8	5,300	51	Macrocytic hyperchromic
2	4/10	26.80	9.49	283	1.35	5.7	1,600	62	Macrocytic hyperchromic
3	6/27	21.81	8.50	257	2.43	7.8	3,850	55	Macrocytic hyperchromic
4	5/3	21.55	9.93	216	2.07	7.9	6,200	56	Macrocytic hyperchromic
5	6/25	15.70	8.80	178	2.54	11.6	3,500	62	Macrocytic hyperchromic
6	5/14	13.93	9.86	141	2.70	12.0	6,050	63	Macrocytic hyperchromic
7	6/27	11.88	8.50	140	2.63	10.1	7,400	62	Macrocytic hyperchromic
8	5/16	15.01	9.90	152	1.94	8.6	5,450	69	Macrocytic hyperchromic
9	4/30	9.94	8.14	122	4.47	8.0	7,900	72	Microcytic hypochromic
10	4/21	13.97	10.41	134	2.64	9.1	4,050	72	Macrocytic normochromic
11	5/2	20.51	11.08*	185	2.69	10.4	4,950	67	Macrocytic hyperchromic
12	5/2	20.17	11.08*	182	3.52	10.7	5,950	45	Macrocytic normochromic
13	5/17	17.81	7.14*	250	2.27	8.8	5,300	65	Macrocytic hyperchromic
14	5/17	24.24	7.14*	343	1.60	5.3	2,300	41	Macrocytic hyperchromic
15	5/23	17.39	10.10	172	3.69	8.3	6,900	49	Microcytic hypochromic
16	6/19	11.81	9.86	119	2.00	9.2	7,350	74	Macrocytic hyperchromic
17	6/15	13.06	8.91	148	2.52	6.9	6,500	85	Macrocytic hypochromic, renal insufficiency,
18	4/25	13.27	8.99*	148	4.30	8.6	8,100	73	chronic G.U. infection
19	4/26	26.45	9.85	269	1.02	6.5	2,100	53	Microcytic hypochromic
20	5/14	17.54	9.86	178	3.15	10.7	3,000	64	Macrocytic hyperchromic
21	5/3	20.32	9.93	204	1.85	8.6	4,500	44	Macrocytic hyperchromic
22	5/14	22.78	9.86	231	3.19	8.6	---	34	Microcytic hypochromic
23	6/1	13.60	10.37	131	2.76	11.2	4,450	73	Macrocytic hyperchromic
24	4/17	16.35	11.67	153	1.11	4.6	6,150	75	Macrocytic hyperchromic
25	3/24	21.36	9.19	232	2.56	9.1	5,450	51	Macrocytic hyperchromic
26	4/26	19.38	9.85	197	0.79	3.3	3,250	49	Macrocytic hyperchromic
27	4/21	15.42	10.41	148	4.11	10.1	4,300	62	Macrocytic hyperchromic
28	5/23	14.73	10.10	146	4.20	6.5	6,500	30	Microcytic hypochromic
29	5/23	16.19	10.10	160	2.78	4.9	6,150	48	Microcytic hypochromic

\*Control R. B. J. All other controls L. J. B.

cases where red cell counts are under two million is 228 per cent. As the erythrocyte counts rise to between 2 and 3 million and to above 3 million, the mean falls to 176 per cent and 166 per cent, respectively. A similar comparison between the per cent "normal" phagocytosis and the total number of leucocytes (column 8) fails to show as close a correlation, but the three most leucopenic patients (Cases 2, 14, and 19) have very high phagocytic activities. Moreover, when the total number of neutrophils alone is considered (found as the product of columns 8 and 9), the inverse correlation is more apparent. However, as explained below, there are exceptions to a high phagocytic activity accompanying low white cell counts.

Cases 14, 18, 25, 26, and 29 of Table I deserve special consideration since they show quite dramatically the decrease in per cent "normal" phagocytosis with remission of the anemia (Table II). In fact, the phagocytic activity *falls below normal* in each, except Case 14, when the red cells are approximately normal. These subnormal values are not unexpected since it has been shown<sup>1, 2</sup> that phagocytosis is depressed in experimental animals on deficient diets. Observations<sup>3, 4</sup> on malnourished patients without anemia show also that the phagocytic activity is less in such persons than in "normals." Thus, as the malnourished patients with anemia improve with respect to their anemia, the phagocytic count may become lower than "normal."

TABLE II

(1)	(2)	(3)	(4)	(5)	(6)
CASE	DATE	PER CENT "NORMAL"	R.B.C.	HGB.	W.B.C.
14	5/17	343	1.60	5.3	2,300
	6/20	152	3.24	9.7	5,300
	7/11	151	3.80	10.7	6,300
18*	4/25	148	4.30	8.6	8,100
	4/30	131	4.50	9.4	8,400
	5/19	60	4.92	12.5	6,700
	6/23	38	4.97	14.0	6,000
24	4/17	153	1.11	4.6	6,150
	5/ 2	115	2.90	8.3	5,150
	5/21	72	3.37	8.8	3,550
25†	4/14	171	3.14	10.1	4,400
	4/28	333	1.87	7.6	3,050
	5/24	270	1.82	6.4	5,650
	6/19	51	3.32	11.1	9,250
26	4/26	197	0.79	3.3	3,250
	5/17	288	0.93	4.4	4,150
	5/24	350	1.38	7.0	4,450
	6/23	108	3.22	11.7	4,600
	7/14	51	4.57	12.7	----
29	5/ 2	193	2.82	5.5	3,200
	5/23	160	2.78	4.9	6,150
	6/19	97	4.26	10.9	6,400
	7/11	77	4.18	12.8	----

\*See Fig. 1.

†See Fig. 2.

The changes in the number of white blood cells accompanying the red cell changes are shown in Table II, column 6. In Cases 14, 25, 26, and 29 there is an increase in white blood cell counts as the per cent normal phagocytosis decreases, but in Cases 18 and 24 there is a decrease in leucocyte counts. Thus the correlation between total leucocytes and phagocytic activity does not always hold, whereas no exceptions have as yet been found in the correlation between the degree of anemia and phagocytosis.

Case 26 should be especially noted, for within a period of twenty-eight days (4/26 to 5/24) the phagocytic activity increased from 197 per cent to 350 per cent. During this interval the erythrocyte count rose from 0.79 to only 1.38 millions and the hemoglobin changed from 3.3 to 7.0 Gm. Could it be possible that over a long period with such severe anemia the phagocytic activity increases and provides additional protection from infection? Could not the time factor be important in determining the phagocytic count? In this case, as well as in all others, the remission of the anemia (5/24 to 6/23) was accompanied by an approximate return to white cell activity to normal (or below).

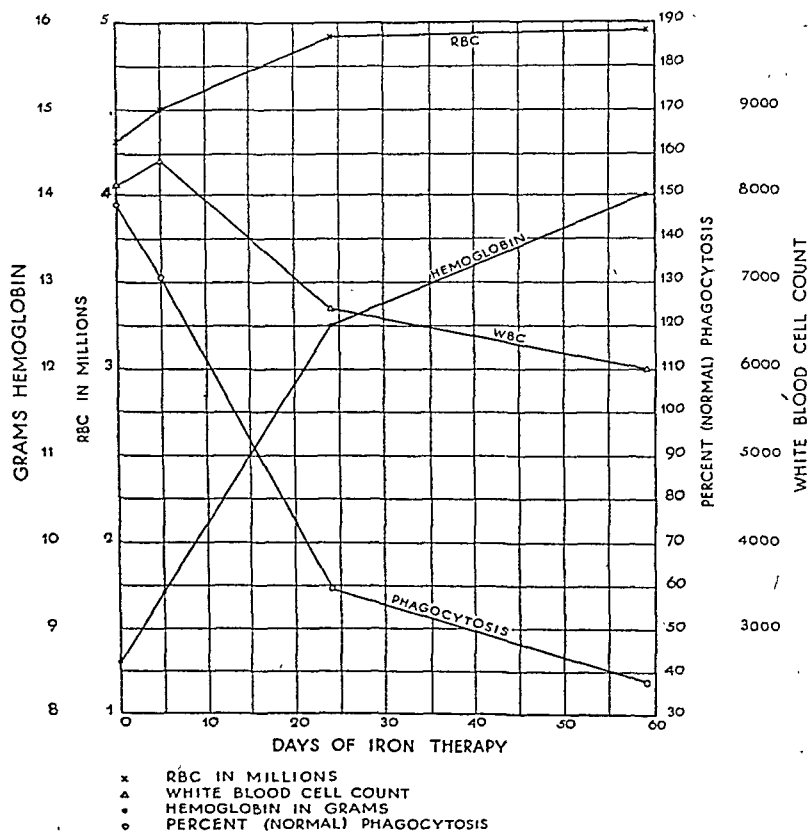


Fig. 1.—Decrease in phagocytosis with therapeutically induced remission in a patient with hypochromic anemia.

The results for Case 18 shown in Table II are presented graphically in Fig. 1. Iron therapy\* was initiated the day the first counts were made and was continued throughout the fifty-nine days covered by the graph. Fig. 1 demonstrates in a striking way that the change in total phagocytosis of the neutrophils is inversely proportional to the change in hemoglobin content of the blood. The two curves in this case are perfect mirror images of one another, while the total erythrocyte curve changes but slightly. The total leucocyte count varies within normal limits and is independent of the other factors shown.

The data of Case 25 are plotted in Fig. 2. The initial drop in erythrocytes, hemoglobin, and leucocytes is accompanied by an approximate twofold in-

\*White's "Mol-Iron" capsules were given three per day. Each tablet contains 3 gr.  $\text{FeSO}_4$  and 1/20 gr. molybdenum.

crease in phagocytosis. Following the administration of one unit of reticulogen per day from the twenty-ninth through the thirty-ninth day, there was a rapid rise in total red and white cells and in hemoglobin, with a marked fall in phagocytosis occurring during the same interval. There is no mirror image between any pair of curves corresponding to that seen in Fig. 1, but in this case both erythrocytes and hemoglobin were undergoing rapid changes. The white blood cells varied inversely with phagocytosis but not symmetrically.

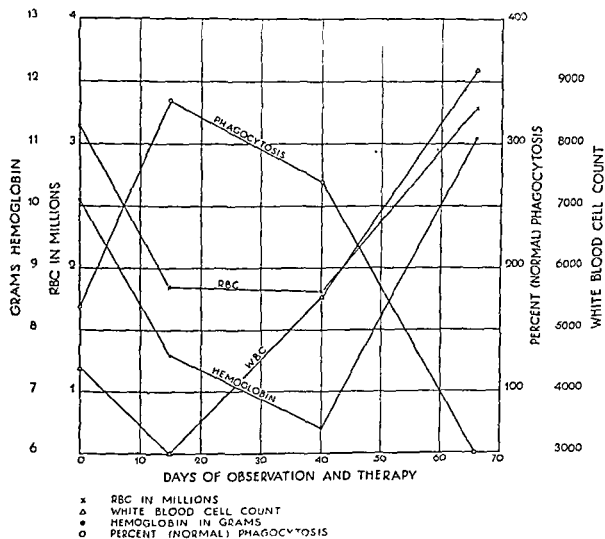


Fig. 2.—Decrease in phagocytosis with therapeutically induced remission in a patient with macrocytic anemia.

#### DISCUSSION

The resistance of all mammals to infections depends upon several factors. These are usually listed as (1) a structural barrier against invasion, (2) humoral defense mechanisms, and (3) phagocytic cells. The leucocytes are probably the final defense against infection, for the ultimate destruction of bacteria is usually accomplished by the leucocytes. Thus, the total effectiveness of the phagocytic cells rests not only upon their number, but also on the activity of each cell. It is therefore important to observe that the phagocytic activity of the neutrophils in anemic persons may undergo as much as a threefold increase. This increase occurs at a time when the total number of white cells usually decreases to a value from one-half to one-third normal. Thus, assuming that the product of the total number of neutrophils and the number of bacteria engulfed per neutrophil reflects the true total phagocytic effort, there is no total loss in the ability of the white cells to overcome the bacteria. Only Case 24 seems to show any significant decrease in the white cell defense mechanism after remission of the anemia, and this may be due primarily to the atypical fall in total leucocytes, for which we have no explanation.

The physiologic basis for the enhancement of phagocytic activity in anemic persons is unknown. At present, experiments designed to provide answers to many questions that naturally arise are in progress. These results will be reported later.

#### SUMMARY

1. In twenty-nine cases of anemia it has been found that the phagocytic activity is from 119 to 340 per cent of "normal." The magnitude of this activity is roughly proportional to the severity of the anemia but fails to follow the leucocyte count in all cases.

2. As the anemia remits, the phagocytosis decreases and may even fall below "normal" values as the red cells approach normal. In the majority of cases there is an accompanying increase in white cells.

3. These studies, which suggest that in anemia a protective mechanism is set up against infections, may offer an explanation for the rather surprising resistance to infections commonly seen in some persons with anemia.

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## FATAL SECONDARY, TOXIC THROMBOCYTOPENIC PURPURA DUE TO SODIUM SALICYLATE

### REPORT OF A CASE

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#### INTRODUCTION

IT IS not surprising that the number of substances responsible for the production of secondary thrombocytopenic purpura is an ever-increasing one due to the discovery of new drugs and chemical agents. The wartime diversion of silk and nylon for military purposes, for instance, was followed by the introduction of "leg-stocking color" cosmetic (Sloan<sup>11</sup>), with the result that a new thrombocytopenogenic substance appeared. In contrast, it is extremely surprising to find an old, well-established, and widely used drug like sodium salicylate among the list of known offenders. Those substances which have been definitely incriminated include sedormid, various sulfonamides, organic arsenicals and bismarsen, gold, quinine derivatives, benzol, smallpox vaccine, chenopodium, ergot, dinitrophenol, iodine, hair dye, snake venom, mercury bichloride, colchicine (Custer,<sup>2</sup> Wintrobe,<sup>12</sup> and Limarzi<sup>8</sup>).

Recently two cases of hemorrhagic complications with death following salicylate therapy were reported by Ashworth and McKemie.<sup>1</sup> In both in-

stances, the predominant change consisted of multiple ecchymoses and hemorrhages throughout the body and especially in the brain. Other than several routine determinations of the red blood count, white blood count, and differential count, no hematologic examinations were performed. Diagnostic procedures usually carried out in hemorrhagic diseases, such as the determination of the clot retraction, bleeding and clotting times, prothrombin time, platelet count, performance of bone marrow examination, and tourniquet test were omitted. In the absence of such data, the causative mechanism of the hemorrhagic phenomena in these cases is completely unclarified.

Nonetheless, on the basis of experimental reports of the occurrence of hypoprothrombinemia during salicylate treatment, these authors conclude that the hemorrhages could be explained on the basis of capillary damage and hypoprothrombinemia. This conclusion is untenable and unwarranted by the evidence which they present.

Search of the literature by the Research Division of the Army Medical Library, personal communications from Dr. Charles A. Doan and Major Richard P. Custer, M.C., and reference to the works of Wintrobe,<sup>12</sup> Kracke,<sup>6</sup> Rosenthal,<sup>10</sup> Limarzi,<sup>8</sup> and Piney and Hamilton-Paterson<sup>9</sup> have failed to reveal a single previously reported case of secondary thrombocytopenic purpura due to sodium salicylate. It is believed that the following case report represents a proved instance of fatal secondary thrombocytopenic purpura caused by sensitization to this drug.

#### CASE REPORT

On Jan. 26, 1944, while overseas, a 35-year-old Negro soldier was admitted to a field hospital with the complaint that the wrists, shoulders, back, and knees were painful and that the wrists had been swollen for about one week. The temperature had been normal and the patient had no symptoms referable to the cardiovascular or genitourinary systems. Joint pains were so severe that he couldn't "even cock his rifle." On physical examination, swelling of both wrists, fusiform swelling of the finger joints, and fluctuation of both knees could be determined. The heart was essentially negative. Roentgenologic examination showed no evidence of bone or joint changes. The sedimentation rate was 24 mm. at the end of one hour. On the basis of these findings, a diagnosis of rheumatoid arthritis was made. No record is made of any medication.

Five days later, Jan. 31, 1944, the patient was evacuated by a British hospital ship to a numbered general hospital. While on board, further studies revealed a sedimentation rate of 24 mm. per hour; white blood cells, 5,800 per cubic millimeter, neutrophilic granulocytes, 65 per cent; lymphocytes, 33 per cent; monocytes, 1 per cent; and eosinophiles, 1 per cent. Urinalysis disclosed a specific gravity of 1,030, acid reaction, 1 plus albumin, and no sugar. A few pus and occasional red blood cells were found, but crystals or casts were not noted. Physical examination corroborated the findings previously described. Heart, lungs, tonsils, and throat were normal. The gingiva, however, was quite infected. At this time, a history of typhoid disease in childhood and a neisserian infection ten years previously was obtained. The diagnosis of multiple arthritis was concurred in by the English medical staff. Sodium salicylate therapy was initiated, a mixture of natrium salicylicum of unknown amount being given every four hours during the ensuing three days on the boat.

Upon arrival at the numbered general hospital Feb. 3, 1944, the patient continued to complain of pain in both wrists, knees, and in all finger joints. The wrist and finger joints were somewhat tender and swollen, but apparently they showed some improvement over their condition at the onset of the disease. Pain was elicited on movement. Temperature elevations of 100.0 to 100.5° F. were noted every afternoon. The sedimentation rate ranged between 22 to 26 mm., the red blood count was 4,090,000 cells per cubic millimeter, and hemoglobin, 75 per cent. Roentgenologic examination of the affected joints disclosed no evidence of bone or joint disease but did demonstrate some osteoporosis. Studies for malaria and sickling were reported as negative. Search for dental foci of infection was fruitless, although several carious teeth were found and treated. The patient was started on sodium salicylate February 4 and received 80 gr. daily until March 8, attaining a total of 2,640 gr.



in thirty-three days. On that date he was transferred to a transport for evacuation to the Zone of the Interior (U. S. A.) and was en route until March 22, when he was admitted to Fletcher General Hospital.

At this time it is important to point out that none of the formal medical records describing the history, physical findings, diagnoses, or medication arrived with the patient, nor were they available during his entire period of hospitalization. After death and subsequent autopsy, however, we entertained considerable curiosity concerning the etiology of the disease and accordingly requested the Adjutant General's Office to forward to us all the military medical records of this soldier. It was only after their receipt that the previously described formal medical record and history of medication with sodium salicylate was obtained.

On admission the patient was unable to give more than a vague description of his joint pains and their onset. Although closely questioned, he had no information to offer concerning the results of the laboratory and roentgenologic examinations. He stated that he had received "pills" in the other hospitals but that he did not know "what they were." He had received no medication on the boat and had continued to experience similar joint pains. He had had two severe chills and knew that his temperature had ranged to 101 and 102° F. daily.

Physical examination disclosed a somewhat emaciated Negro man, aged 35 years, weighing 120 pounds, and measuring 68 inches. Temperature was 102.2° F.; pulse, 100; blood pressure, 100/60; and respiration, 20. Head, neck, eyes, thorax, lungs, and cardiovascular system were essentially normal. No masses, pain, or tenderness could be determined on palpation of the abdomen. The genitourinary and glandular systems disclosed no abnormalities. The shoulder, elbow, and wrist joints, bilaterally, and all interphalangeal joints were extremely swollen, hot, tender to palpation, and painful on passive and active movement. Shortly after admission to the ward, the patient experienced a severe shaking chill.

Urinalysis was essentially negative; the sedimentation rate was 15 mm. per hour; red blood count was 4,180,000; white blood count, 3,000; hemoglobin, 13 Gm.; granulocytes, 63 per cent; and lymphocytes, 37 per cent. The Kahn test and malaria smears were negative. X-ray examination of chest and heart showed no abnormalities. The working diagnosis was possible rheumatic fever or rheumatoid arthritis.

Sodium salicylate medication consisting of 30 gr. daily in conjunction with sodium bicarbonate was started March 22, 1944. Under this therapy the subjective complaints and objective articular findings showed some improvement, although the temperature remained elevated. On March 26 the patient received one multivitamin capsule three times daily. No other drug was given the patient.

On March 31, nine days following the institution of sodium salicylate therapy, the patient began to experience mild epistaxis. Rhinoscopy disclosed many small petechiae of the nasal mucosa. On April 2, two days later, all medication was stopped. A platelet count was 70,000 per cubic millimeter, clotting time was 5 minutes, 45 seconds; bleeding time was 2 minutes, 15 seconds. In ten days the patient received a total of 300 gr. of sodium salicylate.

On April 3, four days after onset of epistaxis, the patient began to complain of pain in his left flank and commenced to show hematuria of ever-increasing degree. At this time ecchymoses were present in the oral and conjunctival mucosae, but none were seen in the skin. The patient's condition rapidly became worse; nausea and vomiting of gross blood occurred and he voided dark, bloody urine containing clots. There was exquisite pain and tenderness in the lower abdomen, especially over the symphysis. In Fig. 1 are shown the results of hematologic examinations at this time. The tourniquet test was strongly positive.

The clinical and laboratory evidence seemed diagnostic of thrombocytopenic purpura. Sodium salicylate as a causative, toxic agent responsible for the onset of this disease was considered, but could not be proved in the absence of evidence of previous medication and sensitization, since, as previously noted, the history of antecedent medication was not at hand during the lifetime of the patient. We were fortunate at this time in having Brigadier General Hugh J. Morgan, M.C., Consultant in Medicine in the Office of The Surgeon Command, in the hospital on an inspection visit. They concurred in the diagnosis of thrombocytopenic purpura and also considered sodium salicylate as a possible etiologic factor, conceding, however, that on the basis of the available evidence, this hypothesis could not be substantiated.

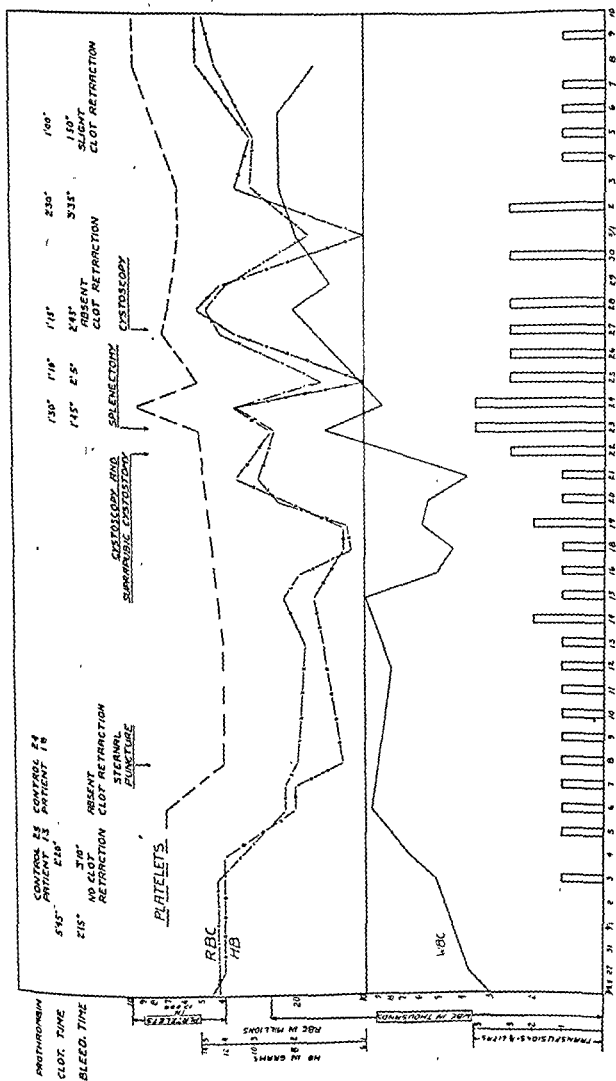


Fig. 1.—This chart shows the consistently low platelet count and absent clot retraction associated with normal clotting and bleeding times. It indicates the progressive, profound anemia and the daily massive transfusions given in the attempt to equalize the hemorrhage. Also illustrated is the absence of thrombocytosis following splenectomy and the leucocytosis resulting from peritonitis. This chart is on a semi-logarithmic scale.

*Course in Hospital.*—The results of serial hematologic examinations are shown in Fig. 1. Also indicated are the daily massive transfusions necessary to combat the severe hematuria, which, on several occasions, threatened to exsanguinate the patient. The platelet level remained consistently low, never attaining values over 90,000 per cubic millimeter.

In addition to transfusions, vitamin B, vitamin C, calcium gluconate, vitamin K, penicillin, snake venom, reticulogen, and even pentnucleotide were utilized without in any way affecting the course of the disease. Blood cultures, serum agglutination tests for typhoid and paratyphoid fever, undulant fever, tularemia, and typhus, and the heterophile agglutination test were negative. On April 16, .2 c.c. of snake venom was injected intradermally and gave a strongly positive reaction.

On April 8 a sternal puncture was performed. This showed a cellular marrow in which no quantitative or qualitative abnormalities of the erythropoietic and myelopoietic elements could be found. Megakaryocytes appeared plentiful but demonstrated qualitative alterations, such as vacuolization and hyalinization of the cytoplasm, absence of granulation, and pyknosis of the nuclei.

The patient's hematuria, dysuria, frequency, and difficulty in voiding increased steadily. On April 21 a cystogram was taken which disclosed six sharply demarcated, spherical, negative shadows both on the right and on the left, averaging about 2 cm. in diameter (Fig. 2), which were interpreted as bladder tumors of unknown etiology. On the basis of the unusual roentgenogram, and because it was thought that the hemorrhage might be arising from the tumors, cystoscopy was undertaken the following day. In addition to multiple areas of submucous hematomas, two spherical neoplasms were found. They appeared to be sessile and solid and showed bullous edema on their surfaces. It was felt that the bleeding was coming from one of the tumors, although the exact site of hemorrhage could not be seen. The lesions were regarded as rare bladder tumors and their removal was advised.



Fig. 2.—Cystogram showing the filling defects caused by multiple, smooth-surfaced, round tumor masses.

Accordingly, a suprapubic cystotomy was performed that day. The bladder was found to be thick-walled and edematous. There were multiple tumorlike projections of the mucosa on both sides of the bladder and several were removed by high frequency current. On cut section they consisted of large, firm, submucous hematomas. Control of the bleeding was only partially successful by electrocoagulation.

On the following day the patient's condition was extremely precarious and the advisability of performing a splenectomy was considered. As an emergency lifesaving measure, therefore, the operation was performed. The results of the histologic examination of the spleen will be considered in connection with the autopsy findings.

There ensued a temporary improvement in the urinary hemorrhage and in the hematologic status, but this was short lived, to be followed by definite signs of peritonitis which

appeared in the region of the cystotomy and which gradually became diffuse. Distention, cardiac embarrassment, and pulmonary involvement became exceedingly distressing; the temperature and white blood count rose steadily and the patient died May 13, 1944.

#### Autopsy Protocol

Autopsy revealed a 35 year-old emaciated Negro man. The skin of the palms and soles and the conjunctival, oral, and nasal mucosa were studded with multiple petechiae. The abdomen was dull to percussion in the flanks and a fluid wave could readily be elicited. Generalized lymphadenopathy was absent.

On incision, the pectoral and abdominal muscles and the fascial spaces contained a few circumscribed areas of hemorrhage. The peritoneal cavity contained 9½ liters of thick, dirty, gray-red, viscid fluid. There was a diffuse plastic fibrinopurulent hemorrhagic peritonitis present throughout the abdominal cavity, lining the visceral as well as the parietal peritoneum and matting loops of large and small intestine together. The dome of the liver and corresponding diaphragmatic surfaces were completely covered by this shaggy exudate. The posterior wall of the bladder was densely adherent to loops of intestine, creating many localized fluid-filled cavities. The liver was small and the anterior edge was thin and wrinkled.

The pericardium and pleural cavities showed moderate effusion. The linings were studded with ecchymoses.

The lungs revealed considerable subpleural and parenchymal hemorrhages. Congestion and edema were prominent.

The heart, aorta, pancreas, and adrenals disclosed no unusual changes.

The anterior edge of the liver was wrinkled and atrophic and the consistency was very soft. The capsule of Glisson was lined by a thick, fibrinohemorrhagic plastic exudate, the surface of which was shaggy and white-gray, while the deeper portions were red, dark, and firm. On cut section the liver parenchyma was moist and of grass green-yellow hue. The lobular markings were not distinct. In several places small hemorrhagic drophike foci were noted.

Many hemorrhages were seen throughout the wall of the gastrointestinal tract. The serosa was lined by a similar plastic exudate. Many large, soft, swollen lymph nodes were present in the radix mesenterii and especially in the para-aortic area at the bifurcation.



Fig. 3.

Fig. 3.—Surface of bladder showing the marked discoloration of the mucosa and the presence of a polypoid tumor.

Fig. 4.—Cross section of the bladder wall showing the thickening and hemorrhagic discoloration of all coats. Arrows indicate the submucous hematomas



Fig. 4.

The kidneys showed no gross hemorrhages. The bladder was removed with the skin and abdominal wall to which it was sutured in toto. The mucosa was dark, discolored, swollen, and nodular (Fig. 3). Lateral to the left ureteral orifice were noted two cherry-sized, smooth-surfaced, dark discolored, soft masses. Lateral to the right orifice, a rough, shaggy, sutured substance defect of the mucosa and the bladder wall could be seen, the surface of which was lined by yellow-gray, necrotic, friable material. Cut sections through the tumor masses revealed large, formed, soft clots in the submucosa which raised the

epithelial lining above the surface (Fig. 4). The entire wall of the organ was swollen, dark, discolored, and hemorrhagic.

The head and brain were not examined.

The spleen was removed twenty days ante mortem. It weighed 115 grams and measured 10 by 7 cm. The capsule was smooth and tense. On section it was dark and of firm consistency and the pulp scraped with difficulty. The Malpighian corpuscles were hardly noticeable.

#### *Histologic Examination of Tissues*

*Lungs.*—Microscopic examination of lungs revealed the presence of many variable hemorrhages throughout the organ, associated with atelectasis and congestion.

*Heart.*—The heart showed no unusual changes.

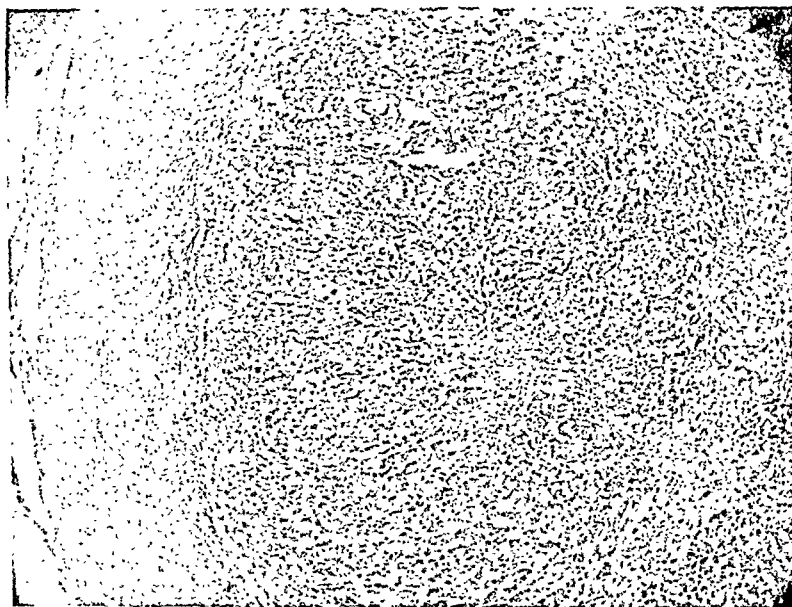


Fig. 5.—Photomicrograph of liver demonstrating the thick plastic exudate of the capsule and the severe toxic necrosis ( $\times 112$ ).

*Liver* (Fig. 5).—The capsule of Glisson was thickened and covered with a layer of organized fibrinohemorrhagic exudate. The subcapsular tissue showed a diffuse round cell and fibroblast accumulation. The liver parenchyma was studded haphazardly by many areas of fresh and old hemorrhages. Some of these were mere punctate foci, while others were quite extended and appeared to be both centrolobular and peripheral. Many hemorrhagic foci showed beginning fibroblastic organization, while others, however, were composed of fresh blood. The liver cells were granular, swollen, and vacular and contained a considerable amount of light yellow-brown pigment. Many of the bile canaliculi were distended with firm, clumping, bile pigment thrombi. There were areas of necrobiosis characterized by the presence of fragments of liver cords and cells, many of which possessed highly pyknotic nuclei. There was a diffuse acute and chronic inflammatory reaction comprising round and plasma cells and granulocytes. The sinusoids were congested, particularly in the mid-zones. The Küpfer and endothelial cells showed a very prominent swelling. Many of the swollen endothelium were lying free within the lumen and were packed with pigment. Sections from various portions of the liver revealed necrotic and hemorrhagic changes ranging from minimal to extensive.

*Pancreas.*—The pancreas was normal.

*Spleen.*—The capsule was intact and of uniform thickness. The trabeculae were thick and fibrous. There was marked reduction in the number and size of the Malpighian bodies, only a few normal nodules being present. The usual lymph follicle was small and possessed a rudimentary germinal center. Its cells were markedly swollen and irregular in size; the nuclei stained poorly and were vesicular in appearance, and the cytoplasm was quite acidophilic. Many small, basophilic particles derived from degenerated pyknotic nuclei

were lying within these swollen reticulum cells. There appeared to be considerable mid-follicular fibrinous exudate (toxic reaction).

The sinusoids were markedly congested. The stroma was dense and filled with solid sheets of large, swollen plasma and reticulum cells. These possessed large, vesicular, lobulated or reniform nuclei which appeared to be somewhat eccentrically located within the cell. The cytoplasm was usually moderately or strongly acidophilic. Large numbers contained a dark brown or yellow, clumpy or granular pigment. Phagocytosis of basophilic particles which might possibly have been derived from necrotic cells, platelets, or extruded erythrocytic nuclei was noteworthy. There was considerable thickening of the walls of the small terminal arterioles. In many cases the lumens were markedly narrowed and, in a few instances, completely occluded.

In occasional sections, areas of coagulation necrosis were present. These foci were diffusely dotted by many small punctate basophilic-stained particles. In the periphery, remnants of cells which were undergoing necrosis were seen. External to this zone there were many large, swollen, histiocytic cells which were choked with small, round, basophilic particles. Large numbers of multinucleated giant cells were seen. These assumed very large proportions of bizarre appearance; many possessed from two to five nuclei which were either lobulated, indented, vesicular, or pyknotic. The cytoplasm was quite acidophilic. Abundant large mononuclear giant cells frequently showing granulation were also present. These elements represented foci of erythropoiesis and myelopoiesis as well as the appearance of megakaryocytes.



Fig. 6.—Photomicrograph of the submucous hematomas of the bladder (X16).

Lymph nodes from the radix mesenterii, para-aortic, and para-pancreatic region were studied. Qualitative alterations were quite similar in all nodes. There was considerable engorgement throughout, the capillaries being particularly distended. Foci of hemorrhage were frequent. There was a network composed of well-developed, fibrous septae which arose in the capsule or hilus and which created a lobular appearance. The usual nodal pattern was absent and lymphocytes were distributed rather evenly. Germinal centers were completely absent and there was no focal accumulation of lymphocytes. Amidst these cells were strewn innumerable large monocytoïd cells possessing eccentric reniform or lobulated nuclei, the chromatin of which was usually reticulated or Rad-kern in appearance. These cells possessed definite cell boundaries; the cytoplasm was usually somewhat opaque and nongranular in appearance. Many had a strong acidophilic cytoplasm. A moderate number of large multinucleated and multilobulated cells were strewn irregularly throughout the tissue. Frequently the giant cells possessed mitotic figures. The peripheral sinuses were not particularly swollen. They contained a moderate number of round and reticulum cells, some of

which were swollen and contained a brown-yellow clumpy or granular pigment. Basophilic nuclear debris frequently appeared to have been engulfed by phagocytic cells.

The serosal surfaces of the gastrointestinal tract were coated by a thick mass of hemorrhagic plastic exudate.

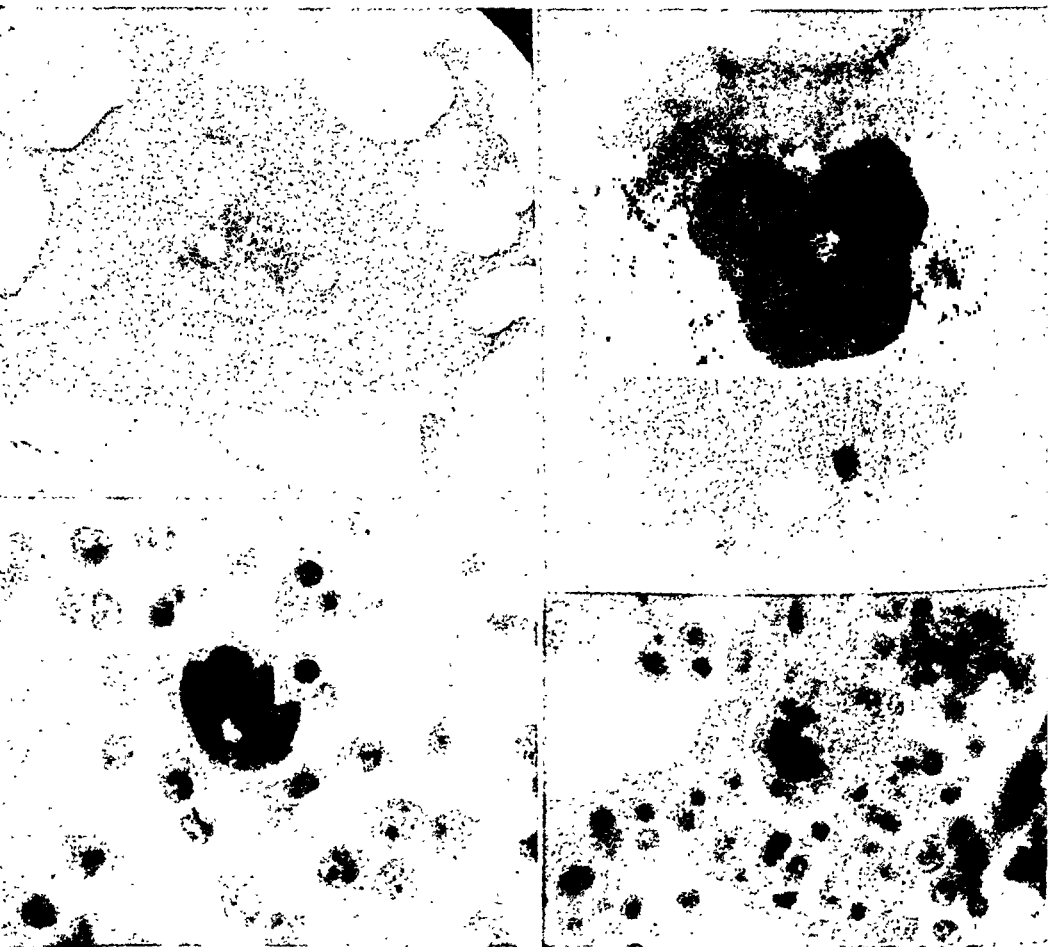
*Kidney.*—There was considerable diffuse, as well as patchy, congestion throughout the parenchyma, especially in the cortex. The glomeruli were considerably engorged but showed no degenerative changes. The tubular epithelium was swollen, granular, and vacuolar and had desquamated in some places, forming solid plugs. In several places dry, contracted, hemoglobin casts were noted. There were many foci of lymphocytic and plasma cell infiltration, especially in the perivascular tissues.

*Bladder.*—Sections through the tumor mass of the bladder wall disclosed the epithelium to be completely absent. The lamina propria was edematous and infiltrated with many large macrophagic cells containing brown pigment. Between the muscle coat and the mucosal layer, there was a large, firm clot showing some degree of organization (Fig. 6). All coats were diffusely hemorrhagic.

*Bone Marrow (Direct Smear and Fixed Specimens).*—Bone marrow consisted of broad sheets of cells with comparative absence of fat. There was considerable congestion of the sinusoids. The cells could be readily identified as members of the granulocytic series, including myeloblasts, a moderate number of myelocytes, and juvenile, stab, or segmented forms.

A.

B.



C.

D.

Fig. 7.—A and B, Photomicrographs of megakaryocytes on direct smear obtained by sternal puncture. These illustrate the severe toxic vacuolization of the cytoplasm ( $\times 1400$ ). C and D, Photomicrographs of megakaryocytes from fixed bone marrow obtained at autopsy. Here the bizarre-shaped, pyknotic nuclei are clearly evident ( $\times 900$ ).

There were, also, considerable numbers of normoblasts and nucleated red cells. Megakaryocytes were present in abundance. These showed considerable evidence of hyperplasia demonstrated by the large, swollen, nonlobulated, juvenile forms. Adult forms could be seen but these disclosed considerable degenerative alterations characterized by the presence of pyknotic nuclei, vacuolization of the cytoplasm, and loss of granulation (Fig. 7 A, B, C, D).

#### DISCUSSION

The diagnosis of thrombocytopenic purpura in this case appears to be well supported on the basis of consistently low platelet counts, absence of clot retraction, somewhat prolonged or normal bleeding time, normal coagulation time, the positive Rumpel-Leed phenomenon, positive venom skin test associated with spontaneous purpura, and free bleeding from the bladder mucosa. The anemia was not out of proportion to the amount of bleeding; there were no pathologic cells in either the blood or bone marrow diagnostic of leukemia or specific anemic states (aplastic anemia, pernicious anemia, chronic hypochromic anemia), and there was no appreciative enlargement of the spleen, further ruling out the group of congestive splenomegalies.

Because of the marked reduction in the number of blood platelets, consideration of nonthrombocytopenic hemorrhagic disorders may be safely omitted. A diagnosis of Schönlein's purpura is not valid, since the arthritic manifestations antedated the hemorrhagic phenomena by several months. Henoch's purpura may also be excluded, since the abdominal pain, epigastric distress, hematemesis, and melena diagnostic of this condition were consistently absent. Vitamin C or K deficiencies, hemophilia, hereditary fibrinogenopenia, multiple hemorrhagic telangiectasia, and hereditary hemorrhagic diathesis are not supported by any evidence on hand.

With the primary diagnosis thus assured, further analysis of the case is mandatory in order further to classify the syndrome as either essential, primary, or as secondary, symptomatic, thrombocytopenic purpura. Our patient was a Negro man, aged 35 years, a fact which poses several preliminary questions of probability. According to Wintrobe, Hanrahan, and Thomas,<sup>13</sup> and Limarzi,<sup>8</sup> essential thrombocytopenic purpura is exceedingly rare among the Negro race. Extremely striking is the high incidence of cases in young female adults. Wintrobe, Hanrahan, and Thomas found that 88.7 per cent of the cases occurred before the age of 24 years. No more than 10 per cent of the cases were over 40 years of age. In their series, Wiseman, Doan, and Wilson<sup>14</sup> found 60 per cent before 21 years and 50 per cent before adolescence.

On the basis of these statistical data, the chances of a male Negro, 35 years of age, suffering from essential thrombocytopenic purpura are reduced. Since the development of essential purpura hemorrhagica is much more common in childhood, the appearance of purpura in an adult should arouse suspicion of a chemical etiologic agent.

The evidence in favor of toxic, secondary thrombocytopenic purpura in our case is quite impressive. This will be presented from the standpoints of the history of exclusive medication with sodium salicylate, the failure of splenectomy to cure, and morphologic data.

*The History of Exclusive Medication With Sodium Salicylate.*—Sodium salicylate was started Jan. 31, 1944, and consisted of a mixture of unknown amount which was given every four hours for three days on the British hospital ship. After admission to a numbered general hospital, this therapy was continued, 80 gr. daily, from Feb. 4, to March 8, 1944, which totals 2,640 gr. in



thirty-three days. From March 8 to March 22 the patient was enroute from overseas to Fletcher General Hospital and no medication of any kind was given. The patient was started on sodium salicylate March 22, with a daily dose of 30 gr., and this medication was continued until and including April 1. A total of 300 gr. were given in this ten-day period. No other drug except an occasional multivitamin capsule was given the patient.

It is significant that the patient was exposed to a comparatively large amount of sodium salicylate (2,640 gr.) for thirty-three days without demonstrating any hemorrhagic phenomena. This was followed by a drug-free phase of fifteen days, at the end of which he was once again exposed to the same noxious agent. Nine days after onset of this second exposure, the bleeding began. This behavior is so similar to the course of events in "serum sickness," for instance, that it is difficult to conceive of a mechanism other than an antigen-antibody reaction of allergic nature. The nine-day period of latency following re-exposure in our case is classical for other allergic states.

A similar behavior has already been recorded in a number of cases of thrombocytopenic purpura due to Sedormid, a notorious offender.<sup>5</sup> Although large amounts of this drug had been taken for a prolonged period of time (several months) without untoward effects, injection of a comparatively small dose after a short period of abstinence was followed by purpura. The deliberate administration of small amounts of Sedormid and of organic arsenicals<sup>4</sup> caused the development of purpuric and ecchymotic lesions within a short time, and, in the case of arsenicals, varying degree of shock. Peripheral thrombocytopenia has been observed in experimental anaphylactoid shock,<sup>7</sup> although the degree of platelet reduction had been variable. Allergic sensitivity to vegetable foods, snake venoms, and insect bite has been incriminated in purpura. To recapitulate, it is probable that the patient became sensitized to the antigen (sodium salicylate) during his hospitalization overseas, which stimulated the production of sodium salicylate antibody in his serum. Upon readministration of the specific antigen, the allergic reaction occurred nine days after onset of exposure and expressed itself in the form of a hemorrhagic diathesis.

*The Failure of Splenectomy to Cure.*—The megakaryocytes in bone marrow examination were found in ample numbers. At the time of examination, the significance of their qualitative morphologic changes was not appreciated and, thus, on the basis of these alterations, no objection could be raised against splenectomy. Personal communications from Dr. Charles A. Doan, following the fait accompli, enlightened us as to the correct interpretation of these abnormalities and furnished additional evidence for the belief that this was a secondary thrombocytopenic purpura. In the light of his experience, vacuolated cytoplasm, pyknotic nuclei, and absence of granulation constitute evidence of a toxic effect. In addition to these changes, the presence of metaplastic foci of megakaryocytes in the spleen is further substantiation of the toxic genesis.

*Morphologic Data.*—Failure of splenectomy to cause cessation of the hemorrhages is introduced as valid evidence to support the diagnosis of secondary toxic thrombocytopenic purpura due to sodium salicylate, since it has been definitely established that this operation is particularly effective in controlling the hemorrhages of primary thrombocytopenic purpura.

Doan,<sup>3</sup> and Wiseman, Doan, and Wilson<sup>14</sup> stress the contraindications of splenectomy in cases of doubtful diagnosis, cases presenting a recent history of

contact with certain drugs, cases occurring during the actual or convalescent stages of all infectious diseases, and cases in which the bone marrow shows depletion of the megakaryocytic content associated with morphologic abnormalities. These mandates have secured universal acceptance. In our case, the absence of a history of previous medication with sodium salicylate, the lack of a precedent of the thrombocyte depressing action of this drug, and the unawareness of the significance of the morphologic changes in the megakaryocytes prevented the establishment of proof of an allergic reaction. In view of the fact that the prognosis was considered grave, and because 20 liters of blood had been given without improvement, splenectomy was performed as a last resort.

#### CONCLUSIONS

1. A case of fatal secondary toxic thrombocytopenic purpura due to sodium salicylate is presented.

2. The causal relationship is based on the proved history of sensitization to sodium salicylate, the absence of exposure to any other chemical or infectious agent, the morphologic characteristics of the megakaryocytes, the metaplastic appearance of these elements in the spleen, and the failure of splenectomy to improve a thrombocytopenic purpura.

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# THE MORPHOLOGY OF THE ERYTHROGENIC RETICULUM OF THE BONE MARROW IN ADDISON-BIERMER'S DISEASE IN RELAPSE AND EARLY REMISSION AS SEEN BY THE IMPRINT METHOD

WITH REFERENCE TO THE MYELOGENIC AND MEGAKARYOCYTOGENIC RETICULUM

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MINNEAPOLIS, MINN.

THE morphology of the bone marrow organ in Addison-Biermer's disease (addisonian pernicious anemia) has received considerable attention with the advent of intravital bone marrow studies for diagnostic, therapeutic, and prognostic purposes. A critical analysis of the pros and cons pertaining to the character of the erythro-, myelo-, and megakaryocytopoiesis revealed that the structural pattern of the reticulum has been little studied. The reasons for the treatment of the reticulum as a minor subject are (a) the widespread presumption that the absence of the so-called "antipernicious anemia principle" (erythrocyte maturation factor) brings about a "maturation arrest" of the erythroblast (megaloblast), myeloblast, and megakaryoblast, (b) the use of the term "megaloblast" to designate the earliest recognizable erythroblast and the belief of Sabin<sup>10</sup> that this erythroid cell is morphologically identical with the basophilic "megaloblast" in the marrow affected by Addison-Biermer's disease, and (c) the widely accepted concept of Doan,<sup>4-6</sup> that the erythroid cell is derived from the endothelium of "intersinusoidal capillaries." These factors have led to diametrically opposed opinions as to the type of erythroblasts present in the normal marrow and in the marrow affected by Addison-Biermer's disease<sup>9, 11</sup> and as to the mechanism by which normal erythropoiesis is re-established.

In order that the reader may more fully appreciate the significance of the statement just made, a few of the most recent discourses pertaining to the type of erythropoiesis present in the marrow in relapse and early remission of Addison-Biermer's disease will be briefly discussed. Schartum-Hansen<sup>20</sup> believes that the promegaloblast is the precursor (primitive form) of the macroblast (pronormoblast of other authors). Under specific therapy the promegaloblast differentiates into a macroblast and the latter into a normoblast. According to Lambin and DeWeerd,<sup>14</sup> the promegaloblast may either differentiate into an intermediate proerythroblast "erythroblastes intermediaires" which matures into a polychromatic megaloblast, and at this maturation stage the identity of the cell is lost, or the promegaloblast may develop directly into a pronormoblast and normoblast, respectively. Davidson and associates<sup>1, 3</sup> refer to the promegaloblast as type I erythroblast, which, according to these investigators, includes the proerythroblast and primitive megaloblast of other authors. This type I cell, which is present in the normal bone marrow, is increased in addisonian pernicious anemia. Under specific therapy it develops into a type II (morphologically similar to the intermediate erythroblast of Lambin and De-

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Weerdt) and subsequently into a type III erythroblast, that is, the normoblast. Wilton<sup>22</sup> presumes that the development of a megaloblast is the sequela of an abnormally slow maturation of a cell he designates "reticulo-erythroblast or erythro-myeloblast," morphologically identical with the "granulo-myeloblast" but biologically different. He believes that megaloblastosis is the expression of a disturbed tissue development. The alteration of the nuclear morphology (nuclear maturation arrest) would be proportional to the degree of the pathologic physiology. Wilton assumes that liver therapy accelerates the maturation process and restores normal erythropoiesis. Vischer<sup>24</sup> is of the opinion that the megaloblast is not transformed into a normoblast. This view is shared by Limarzi<sup>14</sup> and Rohr.<sup>18</sup> Koller<sup>13</sup> believes that a "mehrkerniger Megaloblast" or "Giantoblast" gives rise, by the process of heteroplastic division, to as many as three normoblasts. The latter cell type is further provided by small megaloblasts, "Übergangsformen," which develop into normoblasts, whereas other normoblasts are produced by homoplastic development of retained erythroid cells. Jones,<sup>8-11</sup> who derives the erythroblast from the myeloblast and reticulo-endothelium, believes that the vast majority of megaloblasts can be traced to the myeloblast. He states that the "megaloblast" is a pathologic cell and that the normoblasts which constitute part of the conversion pattern are derived from remnants of the normoblastic series and by heteroplastic development from the reticulum, the myeloblasts, or both. Limarzi and Levinson<sup>15</sup> hypothesize that the rapid transformation of the megaloblastic to normoblastic marrow is caused by multipolar mitosis of a stem cell (which, according to their hypothesis, originates from the reticulum) and by large multinucleated erythroid cells which give rise to normoblasts only. Doan<sup>25</sup> is of the opinion that a maturation arrest of the megaloblast occurs because of lack of the erythrocyte maturation factor which, in turn, results in a nonutilization of iron considered essential for the maturation of erythroid cells. Administration of specific therapy results in the utilization of iron and production of early and late erythroblasts and normoblasts, respectively. Watson and associates<sup>21</sup> suggest on the basis of protoporphyrin studies that the reticulocytes appearing after liver therapy may be derived from megaloblasts.

I<sup>24</sup> have stated elsewhere my opinion (a) that the promegaloblast is a pathologic cell and is derived from a diseased reticulum and (b) that this early erythroblast does not give rise nor develop into a pronormoblast or basophilic erythroblast. Furthermore, I expressed the belief that the pronormoblast and vice versa. Moreover, I expressed the belief that the promegaloblast characterizes the marrow morphology of patients with Addison-Biermer's disease per se and biologic variations\* of this hereditary and complex clinical entity. This concept is strengthened by the observations presented in this paper.

The preceding brief outline of the most recent hypotheses regarding the character of the physiologic and pathologic erythropoiesis and the process by which the latter state subsequently is corrected shows that the subject is of a highly controversial nature.

As to the morphology of the reticulum, Schulten<sup>26, 27</sup> observed that the "Retikulumzellen sind meist sehr zahlreich und gross." Vischer<sup>24</sup> remarks that the reticulum is "einem eigentümlichen Mark-Wucherungsprozess unterworfen." Liver therapy, according to him, brings about a retrogression of the reticulum hyperplasia. Rohr<sup>18</sup> speaks of a "stark proliferierenden Retikulum." Jones<sup>11</sup> suggests a probable involvement of the reticulo-endothelium.

\*Group of diseases genetically related to Addison-Biermer's disease. Such a variable may be identified by the morphology of the reticulum and presence of promegaloblasts of the type described in this paper.

It is the purpose of this paper to show (a) that Addison-Biermer's disease (addisonian pernicious anemia) involves the reticulum, (b) that the reticulum is pathologically activated, (c) that the lack of the liver principle does not cause a maturation arrest of the normal erythroblast but that a pathologic erythroid cell the "promegaloblast" is formed, (d) that organotherapy (specific therapy) affects the reticulum, and finally (e) to re-emphasize the close relationship between the reticulum and the so-called stem cells, the erythroblast, myeloblast, and megakaryoblast, by showing that these cells retain structural patterns identical with the activated reticulum which proliferated them.

#### MATERIAL

One hundred two patients (53 men and 49 women) with uncomplicated Addison-Biermer's disease (addisonian pernicious anemia) in relapse served as subjects. These individuals were under the care of the medical service of the Minneapolis General Hospital. With the exception of four patients who showed free hydrochloric acid in the gastric juice after histamine stimulation,\* all subjects presented clinical and hematologic features characteristic of Addison-Biermer's disease in relapse. The insidious onset of this hereditary and complex disease makes it practically hopeless to fix the date of the first symptoms, the number of attacks, and the spontaneous remissions preceding the progressive downhill course which forced the patient to seek medical attention. For practical and statistical purposes, these patients were therefore classified "first admissions," meaning that none of them was under the observation of a physician and had no specific therapy previous to hospitalization. The majority of the group was of Scandinavian stock. The age of the patients ranged between 29 and 88 years, with a mean of 59.3 years for the male and 65.8 years for the female. The mean age for the entire group was 62.5 years. The circulating erythrocyte quantity ranged between 480,000 and 2,800,000 cells per cubic millimeter. The mean corpuscular volume was between 98.6 and 160 cubic microns. The reticulocyte percentage was between 0.3 and 1.5 per cent. The sternal part of the bone marrow organ was hyperplastic in the majority of the patients. Promegaloblastosis was conspicuous in all marrow specimens. The sternal marrow was re-examined twenty-four, thirty-six, forty-eight, and seventy-two hours after specific therapy had been instituted. The distribution of therapy was as follows. Sixty patients received parenteral liver extract, 1 U. S. P. unit, and ten subjects, 15 U. S. P. units daily. Twenty patients were given Ventriculin,† 40 Gm., equal to 1 U. S. P. unit daily. Seven received the juice of one pound of raw liver daily. Five patients recovered from the anemic state without specific therapy (spontaneous remission). These patients served as controls. Another bone marrow aspiration was done on these control subjects at the peak of reticulocytosis to supplement the specimen obtained on admission.

#### NOMENCLATURE

The term "relapse" connotes the characteristic bone marrow morphology and peripheral erythrocyte quantity of less than 3.0 millions per cubic millimeter. The terms "early remission" and "late stage of early remission" are used

\*I am aware of the accepted medical dictum "no anacidity, no addisonian pernicious anemia." I do not believe in such a restricted limitation as far as biologic processes are concerned. Modifications occur in other clinical entities; thus biologic variations should be expected in this disease also.

Over a period of from one to two and one-half years secretion of free hydrochloric acid ceased in all four subjects. The fact that achlorhydria developed and symptoms of relapse occurred when specific therapy was withdrawn shows clearly that the concept of "biologic variations" is correct.

†Parke, Davis & Co., Detroit, Mich.

for the healing phase, that is, adjustment of the pathologic state of the marrow organ toward normality under specific therapy or by means of spontaneous elaboration of the liver principle. The term "reticulum" is applied to quiescent totipotential mesenchymal cells (lymphoid reticulum) and activated reticulum. The terms "erythrocytic reticulum, myelogenous reticulum, and megakaryocytic reticulum" denote activated reticulum which, from the morphologic point of view, is distinctly associated with the proliferation of erythroblasts, myeloblasts, and megakaryoblasts. The term "erythroblast" is used in a collective sense and covers any nucleated erythroid cell in both the normal and pathologic marrow organ. The term "promegaloblast" as defined by Naegeli is applied to the earliest recognizable pathologic erythroblast. The term "pronormoblast" is applied to the earliest recognizable normal erythroblast. The term "myeloblast" as defined by Naegeli is used for the earliest recognizable myeloid cell, and the term "megakaryoblast" is applied to the stem cell associated with thrombocytopoiesis.

#### METHOD FOR OBTAINING GROSS MARROW UNITS FOR IMPRINT AND HISTOLOGIC PREPARATIONS

A detailed account of a method for obtaining gross marrow units from the human sternum has been published elsewhere.<sup>23</sup> Until the inexperienced investigator is familiar with the material from which he is to make "key" imprint and histologic preparations for the purpose of studying the morphology of the reticulum, the aspirated sternal marrow should be gently mixed with finely powdered commercial heparin.\* Key imprints are made without the benefit of an anticoagulant from gross marrow units immediately when aspirated. The aspirated marrow is spread thinly over a paraffin-covered glass plate. A large gross marrow unit is picked up with the flat cut end of a wood applicator. Care must be taken that the tissue be only slightly touched and that it be ever so slightly spread for several millimeters on a chemically clean glass slide. This procedure is repeated until several rows of serial imprints of the marrow tissue have been made.<sup>25</sup> The opposite cut end of the wood applicator is used for picking up another gross marrow unit. This method requires some skill and speed because the gross marrow units must be picked up and the imprints made before a coagulum is formed. The method just described produces a minimum degree of cellular distortion; that is, delicate structural details are preserved. The use of an anticoagulant, however, permits not only transportation of the marrow specimen to the laboratory, but also the selection of gross marrow units suitable for imprint and histologic preparations to be done in a leisurely manner. While the photomicrographs which accompany this article were made from gross marrow units not exposed to an anticoagulant, it has been my experience that for as long as thirty minutes no appreciable changes occur in the architecture of the units and nuclear morphology if the marrow specimen has been kept fluid by heparin as recommended in this paper. It is suggested that the effect of new anticoagulants on the cellular morphology be compared with that of a key imprint. The imprint preparations are stained according to a method published elsewhere.<sup>21, 25</sup> Briefly the procedure is as follows:

1. Whip preparation through the air to facilitate drying.
2. Bring the slide in a horizontal position.
3. Place 0.5 c.c. of Wright stain on the slide and permit the dye to act for two minutes.

\*Commercial grade derived from dog liver (Lots 151 and 152) is recommended. Made by Hyson, Westcott and Dunning, Baltimore, Md.

†Only a certified stain should be used and made up according to directions on the label.

4. Add 2 c.c. of distilled water (pH 6.0 to 6.4). Place a paraffin-coated wooden applicator horizontally over the fluid surface (long axis of the slide) and keep the mixture in motion by removing and reapplying the applicator to the surface of the staining mixture. Repeat this step until stain and diluent are well mixed.

5. Permit the stain to act for from five to ten minutes.

6. Rinse the stain off with distilled water.

7. Dip the slide for several seconds in Schleicher's decolorizer.\*

8. Wash well under running distilled water or two changes of distilled water.

9. Check staining under the microscope while preparation is still wet.

10. Let the preparation dry by air.

The gross marrow units selected for histologic preparations are fixed in a mixture consisting of physiologic saline, 90 c.c., and neutral formaldehyde, 40 per cent solution, 10 c.c. Helly's fixing fluid may be used. Fixation in the latter fluid should not exceed one hour. The units should be serially sectioned about 5 microns in thickness. The sections may be stained either with azure II and eosin, hematoxylin and eosin, or any other preferred routine staining method.<sup>26</sup>

#### GENERAL CONSIDERATIONS PERTAINING TO THE STUDY OF ACTIVATED RETICULUM

While the nature of the mechanism that initiates the development of a quiescent reticulum cell into an erythroblast, myeloblast, or megakaryoblast is still unknown, it appears from the study of activated reticulum that the stimulus is a "specific." When presumably the substance becomes structurally different, or is quantitatively, qualitatively, or both, deficient, the morphology and functional state of the reticulum undergoes conspicuous changes.

Morphologic variations of activated reticulum may be encountered in the physiologic bone marrow organ. The presence of some malformed reticulum should be expected in a structure as complex as that of the bone marrow organ. It is obvious, therefore, that the investigator should be well acquainted with the variations of the morphology of the normal reticulum and artifacts created by the method employed in order that he be able to segregate the aforementioned structural deviations from those caused by Addison-Biermer's disease.

It became apparent from the study of normal and pathologically activated reticulum by the imprint method that the erythrogenic, myelogenic, and megakaryocytogenic reticulum cells have distinct patterns which appeared to be sufficiently constant to permit an observer to distinguish between the various types of reticulum. The pathologically activated erythrogenic reticulum may be divided into three arbitrary patterns which, like all biologic phenomena, are subject to variations. Although the variability does not deviate to such a degree from the standard patterns described here that difficulties arise regarding a clear-cut separation of the patterns, the latter are (a) the morphology of the erythrogenic reticulum in relapse, (b) the morphology in early remission, and (c) the morphology in the late stage of early remission.

While a detailed description of the structural pattern of the erythrogenic reticulum is given in this paper, it should encourage the observer to "see" variations of morphology rather than to fix a too limited pattern in his mind because even the experienced morphologist at times may have trouble classifying activated reticulum according to a static pattern, particularly when the pathologic process is very severe.

*Acetone	0.5 c.c.
Methyl alcohol	5.0 c.c.
Distilled water	100 c.c.

## THE MORPHOLOGY OF THE ERYTHROGENIC RETICULUM IN RELAPSE

The activated erythrogenic reticulum in relapse presented in general the morphology shown in Fig. 1, A. Comparison with the normal erythrogenic reticulum, Fig. 4, A (assumed to be at the same developmental stage), reveals several conspicuous differences in morphology. The chief features are the greater size of the reticulum syncytia and particularly the pattern of the nuclei. Promegaloblasts are in the process of proliferation, are attached to, or are in the immediate proximity of the erythrogenic reticulum. The lower arrow in Fig. 1, A, points to a reticulum nucleus accumulating a cytosome. The nucleoli are not conspicuous. The latter structures are not easily demonstrable because of their presumed fluid state and variability of compressed chromatin about these complex structures. The nuclear chromatin has a fine granular appearance and is uniformly distributed throughout the well-defined parachromatin. The nucleus by its pronounced affinity for basic dyes is sharply outlined against the forming cytosome which ranges in hue from medium to light blue. A nuclear envelope (membrane) is not particularly discernible. It is believed that because of accelerated anabolic and catabolic metabolic processes the envelope is neutral and thus fails to stain distinctly. A perinuclear zone and Hof, whose purpose seems to be to permit a free exchange of metabolites between the cytosome and nucleus, and, more indirectly the nucleoli, appears at this developmental stage. The rate of cellular dehydration influences the amount of precipitation of colloid proteins and other constituents momentarily present within the zone and Hof, and thus granules may or may not be present within these areas.<sup>22</sup>

The constituents of the remaining cytosome are seemingly of a more viscous character than those of the zone and Hof. Thus the cytosome is more or less sharply demarcated from the common cytoplasm of the reticulum. This demarcation is particularly true at the periphery of the cytosome. No correlation by actual measurements could be made out between the severity of the anemic state and the size of the erythrogenic reticulum and nuclei. The tendency toward hugeness of both structures is, however, striking. In general, the erythrogenic reticulum stains more brilliantly and lighter than the normal reticulum and deeper than the myelogenic reticulum. From the morphologic viewpoint it appears that promegaloblasts (Fig. 1, B) are proliferated by the reticulum by means of the heteroplastic process because mitosis of this cell type has not been observed. However, mitosis is the mechanism by which the reticulum multiplies itself, and thus the presence of a mitotic figure indicates that the cell is most likely an activated reticulum cell rather than a promegaloblast. The morphology of detached promegaloblasts is shown in Fig. 1, B. The structural pattern corresponds well with those given in the literature.<sup>9, 17, 23</sup> Attention is directed to the nuclear and cytoplasmic morphology of the promegaloblasts as well as to their size, to the close similarity of morphology of those free cells, and to the cells which are in the process of forming but are still part of the reticulum. In this series the free promegaloblasts have been found to be larger than the pronormoblasts of the physiologic marrow. The cells classified as promegaloblasts range in size from 20 to 27 microns. These dimensions are far above those calculated for the pronormoblasts, which range between 13 and 16 microns.<sup>22</sup> Again no relation could be observed between the size of the free promegaloblasts and the anemic state. Thus it may be inferred that the genetic and constitutional background, at least to some extent, determine the type of cell proliferated.



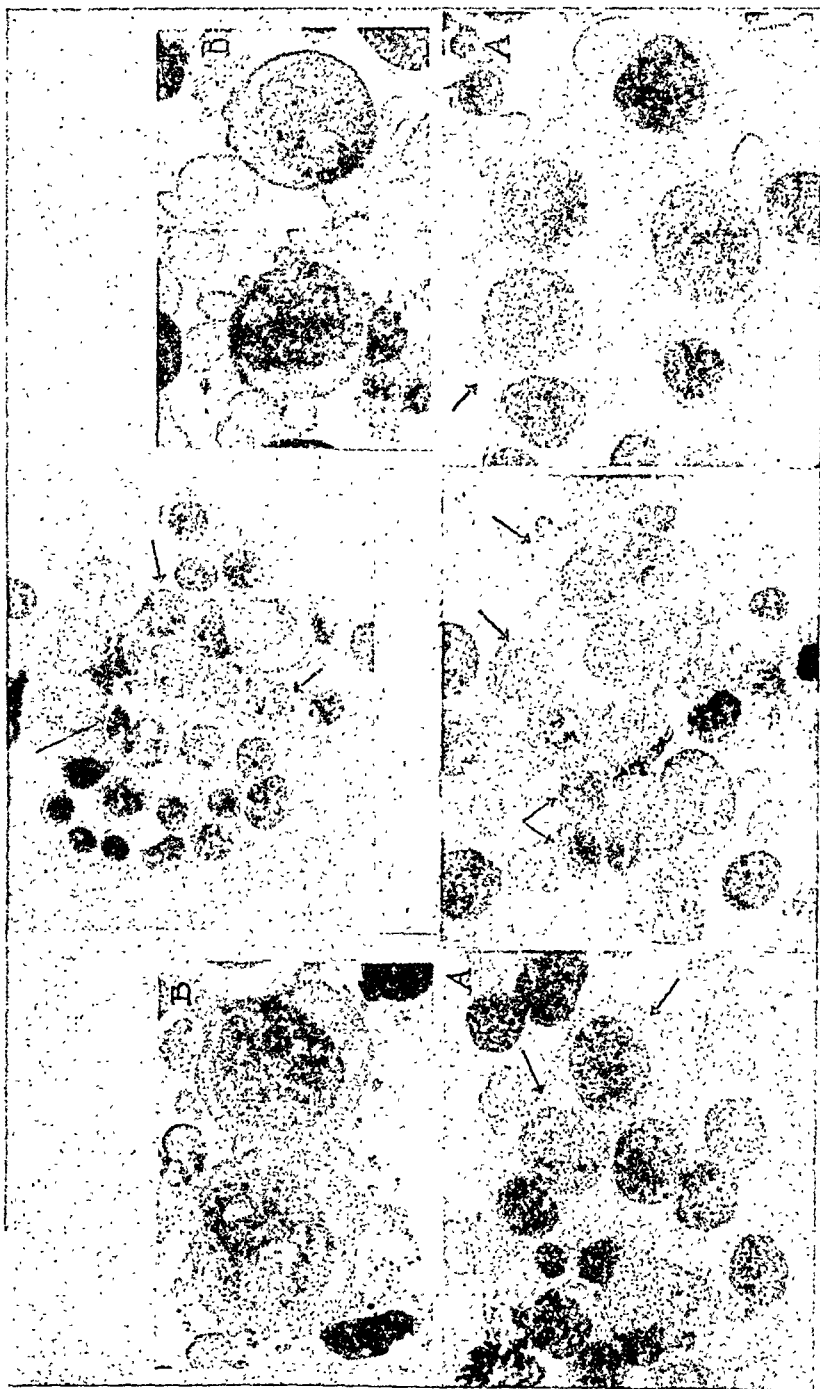


Fig. 1.

FIG. 1.—Addison-Biermer's disease (addisonian pernicious anemia) in relapse. A, Sternal marrow unit imprint showing pathologically activated erythroblastic reticulum. Upper arrow, reticulum nucleus of questionable vitality. Lower arrow, heteroplasmic proliferation of a promegaloblast. B, Free promegaloblasts. Left, early promegaloblast, and right, late promegaloblast.

FIG. 2.—Addison-Biermer's disease (addisonian pernicious anemia), early remission, twenty-four to thirty-six hours after specific therapy. Sternal marrow unit imprint showing pathologically activated erythroblastic reticulum of the intermediate type. Twin arrow, extreme left, late promegaloblast, late telophase. The erythroblast is still attached to an early, promegaloblast by a cytoplasmic bridge. Note the difference of the morphology of the nucleus of the "normoblast-like" megakaryoblasts. The extreme left erythroblast may be difficult to separate from a true normoblast of the same maturation stage. Arrows at the right show an early promegaloblast still attached to the common cytoplasm and a reticulum nucleus accumulating a cytosome.

FIG. 3.—Addison-Biermer's disease (addisonian pernicious anemia), late stage of early remission, forty-eight to seventy-two hours after specific therapy. Sternal marrow unit imprint showing slightly pathologically activated erythroblastic reticulum. Upper arrow, lymphoid reticulum cell. Arrow extreme right, physiologically activated reticulum cell. Lower arrow, slightly pathologic promegaloblast. Note the conspicuous adherence of the erythroblasts to the common cytoplasm.

FIG. 4.—Normal adult human sternal marrow. A, Sternal marrow unit imprint showing physiologically activated erythroblastic reticulum. Arrow points at reticulum nucleus accumulating a cytosome. B, Free pronormoblasts. Left, early pronormoblast, and right, late pronormoblast.

Fig. 2 (Bottom) and Fig. 3 (Top).

Fig. 4.

Occasionally an erythrogenic reticulum fails to develop beyond the stage shown in Fig. 1, A. The common cytoplasm becomes a cytosome of several nuclei. Such a giant multinucleated erythroid mass may degenerate or break up into smaller masses. In a syncytium of the character just mentioned, one or all of the enclosed nuclei may undergo mitosis. The latter phenomenon is known as multipolar mitosis. This process may create the so-called "multinuclear giantoblasts" which, in turn, may or may not give rise to various types of giant erythroblasts.<sup>7, 15, 24</sup>

#### THE MORPHOLOGY OF THE ERYTHROGENIC RETICULUM IN EARLY REMISSION TWENTY-FOUR TO THIRTY-SIX HOUR PERIOD

Conspicuous changes occur in the morphology of the erythrogenic reticulum in the early remission phase induced either by specific therapy or by the mechanism of spontaneous elaboration of the so-called antipernicious anemic principle (erythrocyte maturation factor). In general, the reticulum is of a lesser magnitude—"intermediate reticulum." The size of the nuclei is correspondingly reduced; they are well demarcated but still show a fine granular chromatin. The parachromatin is prominent. The nucleoli still vary in character but are more sharply outlined. The nuclei are surrounded by a well-defined and more intensely blue-stained cytosome which is frequently connected by means of "cytoplasmic bridges" artifacts produced by vacuolation of the common cytoplasm. Promegaloblasts of lesser magnitude either are attached to or are in the immediate proximity of the erythrogenic reticulum. From the morphologic point of view, the cells correspond well to those described by Lambin and DeWeerd as "erythroblastes intermediaires." Mitosis at that developmental stage is common, for heteroplastic cell production is at the minimum, and megaloblasts are now produced by the homoplastic process. At this particular stage the tendency to rupture of the reticulum syncytium and proliferated cells is conspicuously less.

In Fig. 2 is shown an example of an "intermediate reticulum." The top arrow points to a still attached promegaloblast. The extreme right arrow points toward a reticulum nucleus accumulating a cytosome. Two remnants of cytoplasmic bridges (pseudopods) are present. The twin arrow at the extreme left points at a late promegaloblast in mitosis. The erythroblast is still attached to an early promegaloblast. The difference in morphology of the nucleus of the "normoblast-like" megaloblasts is very striking. To classify correctly the erythroblast (at the left bar of the twin arrow) when detached from the erythrogenic reticulum is difficult indeed. Even an experienced morphologist may mistake such a cell for a true normoblast. This type of intermediate reticulum also characterized the marrow morphology of untreated individuals who recovered from the anemic state by spontaneous remission and of patients not included in this series whose erythrocyte quantities were above 3.0 million cells per cubic millimeter.

#### THE MORPHOLOGY OF THE ERYTHROGENIC RETICULUM IN THE LATE STAGE OF EARLY REMISSION FORTY-EIGHT TO SEVENTY-TWO HOUR PERIOD

In Fig. 3 is shown the phenomenal change of the morphology of the erythrogenic reticulum in the late stage of early remission. The dominant erythrogenic reticulum is characterized by a remarkable stability of the common cytoplasm and by a conspicuous coherence of erythroblasts of all maturation stages. The erythrogenic reticulum, however, bears the earmarks of having developed from

reticulum cells of various degrees of vitality. This assumption is believed supported by several conspicuous features: variability of the size of the erythro-genic reticulum, atypical mitosis, and, as the expected sequela, malformed erythroblasts. For example, the malformed pronormoblast in Fig. 3, bottom arrow, is easily distinguishable from the intermediate promegaloblast (Fig. 2, top arrow), although difficulties arise in predicting its developmental potentialities. From a purely hypothetical point of view, this slightly pathologic pronormoblast might give rise to normoblasts. The query arises as to whether this type of erythroblast can develop into an orthochromatic megaloblast in case the effectiveness of specific therapy should be modified in one way or another. The potential presence of pathologically activated erythro-genic reticulum may well account for the recurrence of megaloblasts. In Fig. 3 is further shown one physiologically activated reticulum cell (middle arrow), its nucleus being morphologically identical with the nuclei of the normal erythro-genic reticulum shown in Fig. 4, A, top arrow. Also shown in Fig. 3 is a presumed quiescent (inactivated) lymphoid reticulum cell (top arrow). Physiologic activation of such a reserve cell presumably may occur as early as twenty-four hours (and probably as late as five days) following the administration or spontaneous elaboration of the liver principle. The development of normal erythro-genic reticulum (Fig. 4, A) and pronormoblasts (Fig. 4, B) seems to be dependent upon two factors: (1) the number of quiescent lymphoid reticulum cells which become physiologically activated and (2) the reversibility to normal of only slightly pathologically activated reticulum cells. It is thus obvious that the momentary physicochemical state of the reticulum is of the utmost importance in organotherapy and recovery of the bone marrow as a whole.

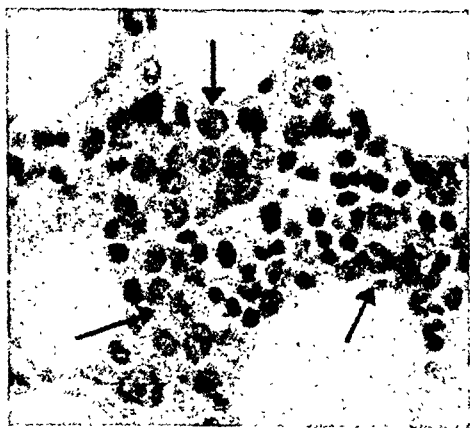


Fig. 5.—Addison-Biermer's disease (addisonian pernicious anemia), late stage of early remission. of a sternal marrow unit. Stained with hematoxylin and eosin. To cells are right arrow, intermediate erythro-genic reticulum. Lower arrow, activated erythro-genic reticulum. Note the normoblasts at the periphery.

In Fig. 5 is shown part of a serial section of a gross marrow unit obtained forty-eight hours after the administration of specific therapy. The top arrow points to an erythro-genic reticulum showing the characteristic relapse pattern. The three cells in the process of mitosis at the periphery are believed to be reticulum cells rather than promegaloblasts. The lower left arrow points toward an intermediate erythro-genic reticulum and the lower right arrow identifies a small and assumed normal erythro-genic reticulum to which are attached pronormoblasts and normoblasts.

The arbitrary division of the erythrocytic reticulum into three functional states according to morphologic patterns seems to furnish a legitimate basis upon which the effect of Addison-Biermer's disease on the reticulum of the bone marrow organ and, by the same token, the effect of specific therapy may be studied. It is believed that ample morphologic evidence has been presented to propose that Addison-Biermer's disease affects the reticulum and that the promegaloblast arises from a pathologically activated erythrocytic reticulum. Administration of the so-called antipernicious anemia principle does not only promote the development of the promegaloblast to a megalocyte (macrocyte of other authors), but also acts specifically upon the reticulum. According to this concept, pronormoblasts (Fig. 4, B) can be proliferated only by an erythrocytic reticulum that is in every respect physiologic.

#### THE MORPHOLOGY OF THE MYELOGENIC RETICULUM IN RELAPSE

The profound effect of Addison-Biermer's disease on the myeloid cells has been thoroughly studied by Tempka and Braun.<sup>29</sup> These investigators observed in the "Vollstadium" (relapse) various degrees of morphologic alterations of the myeloblasts. Analysis of the descriptions given of the morphology of the myeloblasts revealed that activated and free reticulum cells were included in this group of cells. Classification of free stem cells is dependent upon the knowledge of the morphology of the developing myelogenic reticulum. No reference is made by Tempka and Braun to the state of the reticulum. Jones<sup>12</sup> and Sharp and associates<sup>28</sup> noted pathologic alterations in the myeloid cells, but these investigators have omitted a detailed account of the structural pattern of the myelogenic reticulum.

In this series the activated myelogenic reticulum has been found to be easily distinguishable from the erythrocytic reticulum because of its less intense affinity for basic dyes, its morphologic pattern, and its larger size. In Fig. 6; A, is shown a characteristic pathologically activated myelogenic reticulum. Enclosed in this syneytium are several inactivated reticulum cells. The nuclei and cytoplasm of the assumed quiescent lymphoid reticulum cells have a striking affinity for basic dyes. The round to oval reticular nuclei contain from one to several inconspicuous nucleoli. The narrow, not well-defined cytosome contains from few to many small red granules and occasionally a few vacuoles. The cytoplasm varies in color from light to deep blue. The lower left arrow points to an inactivated reticulum and the right and upper right twin arrows point to various developmental stages of activated reticulum cells. The nuclei of the activated myelogenic reticulum may reach the size of the erythrocytic reticulum. The chromatin is in the form of fine strands (sieve-like), in contrast to the granular form characteristic of the nuclei of the erythrocytic reticulum. The nucleus may show from one to several light blue nucleoli. The latter are sharply outlined by compressed chromatin during the differentiation process toward the myeloblast stage. The cytoplasmic mass is less bluish than the common the myeloblast stage. The cytoplasm of the erythrocytic reticulum at the same developmental stage. The cytoplasm of the erythrocytic reticulum is, therefore, not very discernible. The type of myelogenic reticulum described seems to proliferate pathologic myeloblasts. An example of the latter cell type is shown in Fig. 6, B.

In Fig. 6, C is shown an intermediate myelogenic reticulum which characterized the late stage of early remission of Addison-Biermer's disease. While this type of activated myeloid reticulum is conspicuously reduced in size and apparently is also functionally improved, it still proliferates more or less

pathologic myeloblasts. For comparative purposes, a representative of the normal type of myeloblasts proliferated by remnants of, or newly physiologically activated, myelogenous reticulum is shown in Fig. 6, *D*. It is as yet little understood why the return to normal myelogenesis lags so conspicuously behind the readjustment of erythropoiesis and, again, why it recovers more slowly than megakaryocytopoiesis after specific organotherapy has been instituted. This phenomenon has been observed by various investigators, but no feasible explanation has been made. The reason for this sequence of recovery may well rest upon the order of development of the three systems in fetal life. The delayed recovery of the myelogenous reticulum may aid in diagnosing the disease when specific therapy has obliterated the pathologic erythropoietic reticulum.

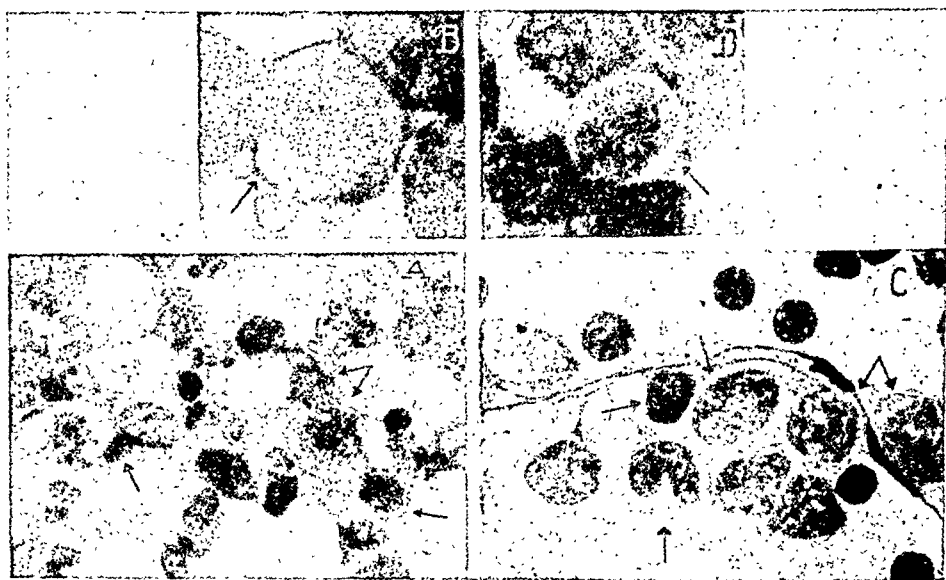


Fig. 6.—Addison-Biermer's disease (addisonian pernicious anemia) in relapse. *A*, Sternal marrow unit imprint showing pathologically activated myelogenous reticulum. Lower left arrow points to an inactivated and arrows at the right point at activated reticulum cells. *B*, Arrow identifies a pathologic myeloblast. *C*, Late stage of early remission intermediate myelogenous reticulum. Top arrow, slightly pathologic reticulum nucleus. Twin arrow, extreme right, shows an early and late myeloblast. Lower arrow points at a giant metamyelocyte. Arrow at extreme left, a lymphoid reticulum cell. *D*, At arrow, normal myeloblast.

#### THE MORPHOLOGY OF THE MEGAKARYOCYTOGENIC RETICULUM IN RELAPSE

Tempka and Braun<sup>29</sup> and Jones<sup>12</sup> have observed various degrees of morphologic alterations in the megakaryoblast, promegakaryocyte, and megakaryocyte, respectively, although the status of the megakaryocytopoietic reticulum is not described. It must be pointed out that recognition of the megakaryocytopoietic reticulum is difficult because it is very small compared to the erythropoietic and myelogenous reticulum. It consisted in this series of from one to two nuclei. Further, it appeared that the megakaryoblast becomes very easily detached from the common cytoplasm. It may be said that in general the nuclei of the megakaryocytopoietic reticulum stain more intensely than those of the myelogenous reticulum. The nuclei are also larger and the chromatin is more coarse and forms irregular masses. The parachromatin is distinct and light red. Nucleoli are difficult to make out because they are generally covered over by chromatin, are small in size, and have a deep blue hue. A nuclear membrane is not discernible. The common cytoplasm is light blue and appears flecky. The nucleus accumulates a narrow cytoplasm when developing toward a megakaryoblast. The

cytosome of the latter is light blue and may contain a few reddish granules. Synchronized with the formation of a cytosome, an area develops in the middle of the nucleus which shows affinity for either acid or basic dyes. This vacuole-like structure varies in size in individual megakaryoblasts. The central location of the structure suggests a metabolic unit and gives the nucleus a doughnut appearance. In Fig. 7, *A*, the extreme left arrow points toward an activated reticulum nucleus. Its cytosome is not well defined. The right arrow identifies a megakaryoblast with a doughnutlike nucleus. The cytosome is narrow and still attached to the common cytoplasmic mass. The development of a central area is not a unique phenomenon of the pathologic megakaryoblast because a morphologically identical structure develops in normal megakaryoblasts also. An example of the latter is shown in Fig. 7, *B*.

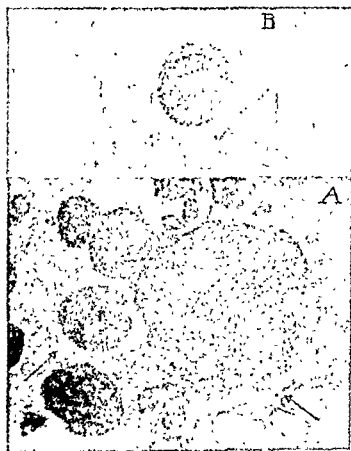


Fig. 7.—Addison-Biermer's disease. *A*, Sternal marrow unit imprint showing a reticulum nucleus. At extreme left, an activated reticulum nucleus. *B*, Megakaryoblast from

anemia) in relapse. *A*, Sternal megakaryocytogenic reticulum. At extreme left, an activated reticulum nucleus. *B*, Megakaryoblast from marrow.

#### DISCUSSION

It is the opinion of the majority of clinical investigators that Addison-Biermer's disease (addisonian pernicious anemia) is a deficiency disease. It is presumed that there is an absence of the intrinsic factor of Castle, although others believe that (a) the gastric principle is not absent but deficient qualitatively or quantitatively, or both, (b) that the intrinsic factor may be present in normal amounts but lack of an extrinsic substance causes a defective production of the gastric factor, and finally (c) that the gastric factor may not be absorbed by the intestines. Whichever theory will become an actuality, the fact remains that the reticulum is affected by the pathologic physiology underlying this hereditary and complex clinical entity.

In my collection of over 7,000 bone marrow preparations obtained during life and covering a large range of hemopathologic states, I have not observed morphologic alterations of the reticulum of the character described in this paper in diseases other than Addison-Biermer's disease and its biologic variables. On

the basis of the 495 marrow specimens obtained for this study, I favor the hypotheses (a) that the administration of specific therapy promotes the maturation process of the promegaloblast, (b) that the proliferation of the promegaloblast is inhibited by the elimination of the mechanism responsible for the pathologic activation of reticulum, (c) that specific therapy eliminates the promegaloblast by improving the metabolism of slightly diseased reticulum, and finally (d) that quiescent reticulum cells again are physiologically activated. It is presumed that these supposed actions of the liver principle are demonstrated by the changes in morphology of the erythro-, myelo-, and megakaryocytogenic reticulum cells. Nevertheless, the fact cannot be overlooked that any rationalization based upon morphologic patterns may suggest, but not necessarily represent, an actual biologic truth. The observation of Watson and associates that protoporphyrin values in Addison-Biermer's disease prior to treatment are low, increase slowly following liver therapy, and reach the highest level some time after the reticulocyte peak, and the presumption of these investigators "that the first mass of reticulocytes appearing after liver therapy may be derived from megaloblasts and may not contain as much porphyrin as those derived from normoblasts" appears to verify the morphologic data presented here. And again the type of erythroblast present in the early and late stage of early remission where the greater number of erythroblasts is made up of "normoblast-like" megaloblasts and of more or less malformed erythroblasts rather than physiologic normoblasts supports the deductions of Watson and associates. Physiologic erythroblasts when present were not prominent in the relapse and early remission phase in this series of cases. Thus it may be stated that the morphology of the reticulum reflects altered physicochemical states. Thus the hypothesis may be advanced that a "functionally altered reticulum is the sequela of a disturbance between the bone marrow reticulum and the substances and organs controlling its physiologic state." The injury to the reticulum by the disease process and the reversibility of the reticulum lesion may be adduced by means of morphologic evidence, although the final evaluation of the damage must await recovery of the bone marrow organ from the effects of the disease.

It is noteworthy that not all of the reticulum becomes diseased. It was observed that quiescent and apparently healthy reticulum cells were situated next to pathologically activated ones. This unaffected reticulum is apparently the reserve tissue upon which, in final analysis, the bone marrow organ reconstructs itself. Thus it may be presumed that, if a certain quantity of reticulum is destroyed by the disease process, the most likely sequela is a permanent hypoplastic state of the bone marrow organ. Furthermore, successive attacks and various types of senile changes within the organ will be a deciding factor regarding revitalization of the organ to a state where the cellular output meets physiologic values. It is believed that this statement is supported by the observation that, in spite of adequate and various therapeutic measures and absence of complications known to interfere with the recovery of the marrow organ, the peripheral erythrocyte quantities may remain between 3.0 and 4.0 millions per cubic millimeter. As a matter of fact, eleven subjects in this series have leveled out at and between these erythrocyte quantities. There is reason to believe that these erythrocyte levels represent the normal productivity of the respective bone marrow organ. In other words, re-establishment of physiologic hematopoiesis is dependent upon (a) the constitution of the bone marrow organ, (b) the degree of injury to the reticulum by a single or repeated attack of the disease, (c) the ability of slightly pathologically activated and quiescent healthy

reticulum cells to respond to the instituted organotherapy or to the mechanism of spontaneous remission. The study of the reticulum of the bone marrow organ affected by Addison-Biermer's disease has suggested that the common denominator for the characteristic erythro-, myelo-, and megakaryocytopoiesis is not the expression of a maturation arrest of normal stem cells but is the product of a diseased reticulum.

The view presented in this paper concerning the attack of Addison-Biermer's disease on the bone marrow reticulum supports the statement of Rohr<sup>12</sup> that "die Verhältnisse wesentlich komplizierter liegen als bisher allgemein angenommen wurde" and the opinion of Naegeli<sup>13</sup> that "der Perniciosa eine ganz charakteristische, einheitliche und scharf ausgeprägte Funktionsstörung des Knochenmarkes zugrunde liegt."

#### SUMMARY

1. The morphology of the activated erythrogenic reticulum cell with reference to the myelogenic and megakaryocytopoietic reticulum cells of the sternal part of the bone marrow organ in uncomplicated Addison-Biermer's disease (addisonian pernicious anemia) in relapse and early remission is presented.

2. One hundred two patients served as subjects. Four hundred ninety-five bone marrow specimens were obtained by means of the sternal aspiration method. Imprints of the gross marrow units were made and stained according to procedures described in this paper.

3. Sternal marrow was obtained before and after twenty-four, thirty-six, forty-eight, and seventy-two hours following the administration of specific therapy. Those patients who showed an intermediate reticulum pattern on admission were used for control purposes, and sternal marrow was obtained at the peak of the reticulocytosis only.

4. Morphologic evidence is presented which appears to favor the hypothesis that the disease affects the reticulum and that the latter is pathologically activated.

5. The morphology of the diseased reticulum in relapse and early remission and of the normal reticulum is sufficiently constant to permit an arbitrary division of the structural pattern of the erythrogenic as well as the myelogenic and megakaryocytopoietic reticulum for the purpose of orientation regarding the effect of the disease and specific therapy on the reticulum.

6. The study of the reticulum suggested that re-establishment of normal hematopoiesis is dependent upon the reversibility of the reticulum lesion, the degree of physiologic activation, and the reproducing capacity of the newly activated reticulum.

7. The common denominator for the pathologic erythro-, myelo-, and megakaryocytopoiesis which characterizes the marrow morphology in Addison-Biermer's disease (addisonian pernicious anemia) in relapse is a diseased reticulum.

8. Under specific therapy or spontaneous remission the diseased reticulum seems to become depleted and thus proliferation of pathologic stem cells ceases. The maturation process of the promegaloblast is apparently promoted, however, developing into a "normoblast-like" erythroblast rather than true normoblast.

9. The close relationship between the reticulum and the so-called stem cells, that is, the erythroblast, myeloblast, and megakaryoblast, is reemphasized by showing that these cells retain structural patterns morphologically identical with those of the respective activated reticulum.



10. It is pointed out that in a large collection of bone marrow specimens obtained during life covering an extensive range of hemopathologic states, morphologically altered reticulum of the character described in this paper has not been observed in diseases other than Addison-Biermer's disease. It is suggested that biologic variations of this hereditary and complex disease may occur. These genetically related clinical entities may be readily recognized by the morphology of the erythrocytic and/or the myelogenic and megakaryocytogenic reticulum as seen by the imprint method.

I wish to thank Dr. G. E. Fahr, Head of the Department of Internal Medicine, Minneapolis General hospital, for his interest in the investigation reported here. The photomicrographs were made by Mr. H. W. Morris, campus photographer, University of Minnesota.

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or decreased. These results indicate that a simple classification into cholericetics and hydrocholericetics does not tell the entire story.

The properties of dog hepatic bile before the administration of atquinol or cinchophen, which might be termed normal or control hepatic bile, have been summarized in Table I. Following the administration of atquinol or cinchophen, six of these properties were altered, the change usually occurring on the first, sometimes the second, day after giving the drug, and by the third day the alterations had returned to, or were returning toward, normal or control levels. In the instance of all properties, there was a change in the mean value after administration of the drugs, but in only six instances were these changes statistically significant. In some cases, the percentage change in the mean was not marked but was statistically significant, while in other cases the percentage change in the mean was considerably greater but the change was not statistically significant. It was therefore decided not to include the mean changes after administration of the drugs listed in Table I but rather simply to indicate if there was a significant change and, if so, what, and to describe the changes quantitatively in the text. The value of tables, charts, diagrams, etc., is to give the reader a quick, *correct* picture of what was found experimentally, and hence it is inadvisable to include changes in the mean which are not significant, as this gives an erroneous impression which has to be corrected by laboriously reading through the text.

TABLE I.—OUTPUT, PROPERTIES, AND COMPOSITION OF NORMAL, HEPATIC BILE COLLECTED FROM HEALTHY, UNANESTHETIZED, DOGS WITH CHRONIC BILIARY FISTULA AND EFFECT OF ORAL ADMINISTRATION OF 0.1 GR. PER KILOGRAM OF BODY WEIGHT OF ATOQUINOL AND CINCHOPHEN

ESTIMATION	UNIT	NORMAL VALUE (MEAN $\pm$ S. D.)	EFFECT OF ATOQUINOL AND CINCHOPHEN
Twenty-four hour volume	Ml. per kilo	7.4 $\pm$ 2.8	Significantly increased
Specific gravity	Gm. per ml.	1.0056 $\pm$ 0.0030	Significantly increased
Relative viscosity	Distilled H <sub>2</sub> O=1	1.322 $\pm$ 0.337	Significantly decreased
Total solids	Gm. per 100 ml.	3.093 $\pm$ 0.124	Significantly increased
Sodium	Mg. per 100 ml.	350 $\pm$ 93	No significant change
Chloride	Mg. per 100 ml.	436 $\pm$ 113	No significant change
Potassium	Mg. per 100 ml.	34.8 $\pm$ 7.0	Significantly increased
Inorganic phosphate	Mg. per 100 ml.	20.6 $\pm$ 11.9	Significantly increased
Total fatty acids	Mg. per 100 ml.	317 $\pm$ 223	No significant change
Total cholesterol	Mg. per 100 ml.	52 $\pm$ 27	No significant change
Ester cholesterol	Mg. per 100 ml.	33 $\pm$ 25	No significant change
Free cholesterol	Mg. per 100 ml.	19 $\pm$ 10	No significant change
Bilirubin	Mg. per 100 ml.	38 $\pm$ 30	No significant change
Bile acids	Gm. per 100 ml.	2.54 $\pm$ 0.78	No significant change

The *twenty-four hour output* of hepatic bile before administering the drugs averaged 7.4 c.c. per kilogram body weight, a value lower than that occurring in man but comparable to that reported in dogs by other investigators. In over 90 per cent of experiments, the administration of atquinol augmented the volume output of bile, the increase during the first day after the drug averaging 84 per cent, the second day 58 per cent and the third day 8 per cent. In all experiments, the giving of cinchophen increased the volume output of hepatic bile, the corresponding figures for the three days being 76, 133, and 27 per cent.

The *specific gravity* of bile was increased in approximately 95 per cent of experiments following the ingestion of atquinol and in approximately 80 per cent following cinchophen. The actual percentage increases in specific gravity were, of necessity, small. The first day after atquinol, the mean percentage

increase was 0.26 per cent, the second day 0.40 per cent, and the third day 0.15 per cent. The corresponding figures following cinchophen were 0.09, 0.46, and 0.25 per cent, respectively.

The *relative viscosity*, as measured with the Ostwald viscosity pipette, was lowered in from 85 to 90 per cent of experiments on the first, second, and third days following the administration of atoquinol, the mean percentage decreases being 6.6, 7.4, and 5.2 per cent, respectively. The relative viscosity was not significantly affected the first day after giving cinchophen, but on the second and third days it was lowered in 75 per cent of experiments, the mean decrease being small, 2.9 and 0.4 per cent, respectively.

*Total solids* were measured by drying aliquots of hepatic bile at 90° C. for 100 hours. The first day after giving the drugs, there was a significant increase in the total solids, an increase which occurred in some 75 per cent of experiments and which averaged 13 per cent following atoquinol and 7.8 per cent following cinchophen. On the second and third days, changes in the value for total solids were not significant following either of the two drugs.

Of the various solids measured, statistically significant changes were found only in the values for potassium and inorganic phosphate, both of which were increased, suggesting that atoquinol and cinchophen have an especial tendency to augment the output of potassium phosphate in bile. On the other hand, there was no significant decrease in the output or concentration of any of the other solids determined, to be described later, so that since the twenty-four hour volume was increased, there was actually an increase in the twenty-four hour output of all of these solids.

The *sodium content* of hepatic bile was determined after the method of Hoffman and Osgood.<sup>8</sup> The control value was 350 mg. per 100 c.c. of bile, and neither the administration of atoquinol nor of cinchophen significantly affected the mean concentration of hepatic bile sodium.

The *chloride content* of dog hepatic bile could not be shown to be affected in a statistically significant manner by the administration of atoquinol or cinchophen, although the mean values were slightly increased. Chloride was estimated by the method of Van Slyke<sup>13</sup> and the control concentration was 436 mg. per 100 c.c., with a standard deviation of 113.

The *potassium content* was determined after the method of Hoffman,<sup>7</sup> and a statistically significant increase could be shown to occur after the use of both atoquinol and cinchophen, the increase being more marked following cinchophen. After atoquinol, the mean percentage increase in hepatic bile potassium was 6.3 per cent on the first day, 10.9 on the second day and 11.8 on the third day. The corresponding values after cinchophen were, respectively, 17.7, 33.1, and 1.3 per cent.

*Inorganic phosphates* were estimated by an adaptation of the familiar Bell-Doisy technique to measurement in a photometer. Both atoquinol and cinchophen produced statistically significant increases in the concentration of hepatic bile inorganic phosphate on the first and second days after administration but not on the third day. Following atoquinol, the mean percentage increase on the first day was 12.6 per cent and on the second day 6.8 per cent; following cinchophen, the percentage changes were considerably greater and averaged 44.3 per cent the first day and 40.9 per cent the second day. An increase in inorganic phosphates was found in between 70 and 80 per cent of experiments following atoquinol and between 80 and 90 per cent following cinchophen.

The *total fatty acid content* of dog hepatic bile was not affected in a statistically significant manner by the administration of either atoquinol or cinchophen. Total fatty acids were estimated after the oxidative method as modified by Boyd.<sup>4</sup> The normal mean was 317 mg. per 100 c.c., with considerable fluctuation in the individual values which was reflected in the high standard deviation as seen in Table I. On the average, atoquinol caused a slight decline in the concentration of total fatty acids and cinchophen a slight increase, but the changes were not marked nor constant and were statistically insignificant.

*Total cholesterol, ester cholesterol, and free cholesterol* were determined by oxidative micromethods as described by Boyd.<sup>4</sup> The values for normal dog hepatic bile, listed in Table I, are lower than those reported by investigators using colorimetric methods for cholesterol because, as is well known, cholesterol estimated in body fluids or tissues by the oxidative technique yields values which average from one-third to one-half lower than those recorded by the colorimetric methods. The administration of atoquinol or cinchophen had no consistent or significant effect upon the concentration of hepatic bile cholesterol.

*Bilirubin* was measured after the method of Thannhauser and Anderson.<sup>12</sup> Normal hepatic bile contained an average of 38 mg. per 100 c.c. of bilirubin and this mean was not significantly affected by the administration of atoquinol or cinchophen.

The *bile acid* content, measured with the photometer and a quantitative test based upon the familiar Mylius-Pettenkofer reaction, had a normal average value of 2.54 Gm. per 100 c.c. and a standard deviation of 0.78. Neither atoquinol nor cinchophen significantly affected the bile acid content of dog hepatic bile.

#### SUMMARY

Atoquinol and cinchophen were given by mouth in a dose of 0.1 Gm. per kilogram of body weight to fifteen unanesthetized dogs with chronic biliary fistula, hepatic bile being collected and analyzed daily.

Atoquinol and cinchophen had a choleretic effect which was present for two days and was declining upon the third day.

There was little quantitative difference between the effects of the two drugs. Both increased the twenty-four hour volume output of hepatic bile, increased its specific gravity slightly but consistently and caused a decrease in its relative viscosity, an increase in the concentration of its total solids, an increase in its potassium content, an increase in its inorganic phosphate content, and no change in the concentration of sodium, chloride, total fatty acids, total cholesterol, ester cholesterol, free cholesterol, bilirubin, and bile acids.

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## ANTICONVULSANT EFFECTS OF STEROIDS

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IT IS known that some steroids are able to influence cellular permeability as demonstrated by the observation of Tipton<sup>1</sup> that cortin depresses the release of potassium from stimulated muscles. Since cellular permeability in the central nervous system is an important factor in determining convulsive reactivity (Spiegel and Spiegel-Adolf<sup>2</sup>), it seemed of interest to study whether some sterols may have anticonvulsant properties. Such an expectation was supported by Selye's<sup>3</sup> observations of hypnotic effects. Preliminary experiments of Spiegel<sup>4</sup> demonstrated that desoxycorticosterone, testosterone, and progesterone are able to raise the threshold for electrically induced convulsions, and experiences of McQuarrie, Anderson, and Ziegler<sup>5</sup> with desoxycorticosterone on two patients with epilepsy seemed to point to a similar direction. In Spiegel's<sup>4</sup> experiments a margin between anticonvulsant and hypnotic dose could hardly be established. It seemed, therefore, desirable to extend these studies to a larger group of steroids, in the hope of finding compounds with a definite margin between these doses.

Similarly, as in Spiegel's<sup>4</sup> previous experiments, the effect upon the threshold for production of convulsions by electric stimulation was determined. This method permits one to ascertain the effect of anticonvulsant agents more unequivocally than the study of antagonistic effects against convulsant drugs such as metrazol (Selye<sup>6</sup>) or cocaine (Aird<sup>7</sup>), since such an antagonism may be due, at least partly, to other factors such as decrease of absorption or of permeability of the cerebral capillaries. The existence of such mechanisms is indicated by the fact that Aird found a protective influence of desoxycorticosterone acetate against cocaine convulsions in doses that are unable to alter the threshold of the brain for production of convulsions by electric stimulation.

We were able to study twenty-nine steroids.\*

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## METHOD

Definite anticonvulsant effects were observed by Spiegel<sup>1</sup> on white female rats. These animals were also employed in Selye's<sup>2</sup> studies of hypnotic effects. In this study, therefore, we used almost exclusively white female rats of from 100 to 150 grams body weight (over 200 animals). Since male rats were resistant even to quantities far beyond the doses effective in female rats and our supply of steroids was limited, we tested these compounds in male rats only occasionally.

The convulsion threshold was determined by using the previously described method of electrical stimulation with the skull intact (Spiegel<sup>3</sup>). The alternating current from the 110-volt, 60-cycle line is reduced by a Variac transformer to the desired voltage (usually 10 volts). After leaving the transformer the alternating current is closed and opened by a relay, the magnet of which is activated by the discharge of condensers (from 1 to 20  $\mu$  F). Since the duration of the activation of the magnet is proportional to the capacity of the condenser, the resistance of the relay circuit being constant, the duration of the stimulation can be varied. The electrodes are placed in the conjunctival sacs. At a certain voltage the duration of the stimulation is increased, step by step, until typical tonic-clonic epileptiform convulsions are elicited. The threshold values are calculated in milliamperes seconds since the voltage and the duration of the stimulation are known, and the resistance can be determined on a Wheatstone bridge, the electrodes remaining on the same place as during the stimulation.

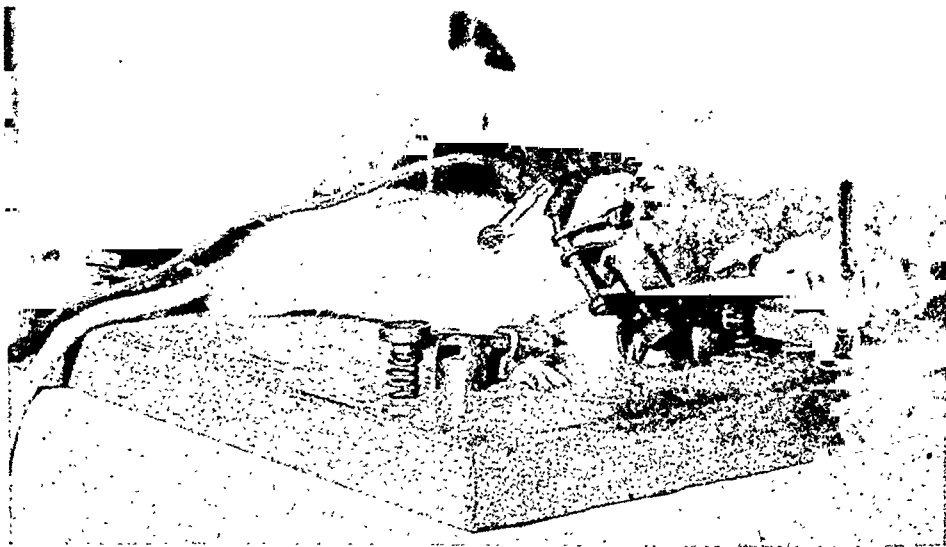


Fig. 1.—Rat board and head holder. Note electrode in right conjunctival sac.

The fixation of the head, application of the stimulating electrodes to the eyeballs, and observation of the convulsions were facilitated by the use of an especially designed head holder (Fig. 1) that fastens the snout, leaving the rest of the head and the eyeballs free. In rats it was of course necessary to apply much smaller electrodes than in rabbits or cats. The electrodes used were slightly concave silver disks with a diameter of 5 mm., which were fitted over the eyeballs. They were held in the conjunctival sacs by small clamps that kept the lids tightly together. With these electrodes the resistance in the circuit usually was between 700 and 1200 ohms. As a rule, at 10 volts a flow of the current from 0.1 to 0.7 seconds sufficed in untreated animals for production of convulsions.

In all experiments the threshold was determined in milliamperes seconds, and the type (extent, localization) and duration of the convulsions were recorded. In order to ascertain the variability of the threshold, at least three tests were made preceding the injection at intervals of from two to three days, and only such animals were used for the injection in which the threshold varied by no more than 30 per cent. The substance under study was applied, as a rule in oily solution, by intraperitoneal injection; occasionally, intramuscular injection of solutions in propylene glycol was used. The convulsion threshold was then determined from one-half to one hour following the injection. The ratio A/B of the convulsion threshold after injection (A) to the average before injection (B) served as a measure of the anticonvulsant effect.

## RESULTS

A substance was considered ineffective, if the ratio A/B was from 1.0 to 1.3 and only slightly effective if this ratio was smaller than 1.5; it was considered anticonvulsant, if this ratio was above 1.5. Applying these criteria, the substances were divided into two groups. In Table I are summarized those which were ineffective or only slightly effective even in relatively large doses.

TABLE I. COMPOUNDS WITH NO, OR ONLY SLIGHT, ANTICONVULSANT EFFECT\*

COMPOUND	DOSE IN MG. PER 100 GM. BODY WEIGHT	CONVULSION THRESHOLD AFTER (A): BEFORE (B) INJECTION
Cholesterol (30 mg. per cubic centimeter olive oil injected in rabbits)	18 to 21 (3 to 5 daily injections)	0.5 to 1.3
Allocholesterol (St)	20 and 50	1
Cholesteryl bromide (St)	20 and 50	1
Epicholestanol (St)	50	1
Stigmasterol (St)	20 and 50	1
Stigmasteryl acetate (St)	20 and 50	1
$\alpha$ -Spinasteryl acetate (St)	20 and 50	1
Ergosterol (St)	20 and 40	1
Ergosteryl acetate (St)	20 and 50	1
$\alpha$ -Ergostenyl acetate (St)	50	1
Dehydrocholic acid (St)	50	1
Desoxycholic acid (St)	20	1
	50	1
$\Delta^5$ -3-Acetoxycholenic acid (St)	50	0.7 to 1.2
Sarsasapogenin acetate (K)	20 and 50	1
Pseudo-sarsasapogenin acetate (K)	20	1
	50	1.2
Diosgenin acetate (K)	20 and 50	1
Pseudo-diosgenin acetate (K)	20 and 50	1
$\alpha$ -Estradiol benzoate (Progynon B; HS)	10,000 rat units	1.3
1.66 mg. per cubic centimeter sesame oil (10,000 rat units)	20,000 rat units	1.1
5 mg. per cubic centimeter (30,000 rat units)	4-8 mg.	1
Theelin in oil (K) 1 c.c. = 10,000 I.U.	10,000 and 20,000 I.U.	1.3
6 ( $\alpha$ ) Acetoxy-progesterone (E) 10 mg. per cubic centimeter; amorphous modification	5 and 10	1
Etio-cholan-3 $\beta$ -ol-17-one acetate (K)	20	1 to 1.1
	20 and 40	1
5-Pregnen-3 $\beta$ -ol-20-one acetate (K)	50	1.0 to 1.6
	20	1
	30	1.3
	50	1.4
5, 16-Pregnadien-3 $\beta$ -ol-20-one acetate (K)	15	1
	20	1.0 to 1.3
	30	1
Stilbesterol 13 mg. per cubic centimeter olive oil	10	0.9 to 1.1
	20	0.7 to 1.0
20 mg. per cubic centimeter olive oil	30	1

\*If not otherwise indicated, all substances were dissolved in olive oil, 20 mg. per cubic centimeter and injected intraperitoneally in white female rats. The source of the material is marked as follows: St, Dr. H. E. Stavely; K, Dr. O. Kamm; HS, Dr. E. Henderson and Dr. E. Schwenk; E, Dr. M. Ehrenstein.



In Table II are shown the steroids with a definite anticonvulsant effect manifested by an increase of A/B above 1.5 and/or by a decrease of the duration of the convulsions, the reaction being reduced in some instances (for example, after injections of progesterone) to single twitches. In Table I cholesterol also is included. We have already found in previous studies<sup>9</sup> that injection or feeding of this compound fails to raise the convulsion threshold. Likewise in Table I are included compounds similar to cholesterol such as allocholesterol, cholesteryl bromide, epicholestanol, phytosterols (stigmasterol, stigmasteryl acetate,  $\alpha$ -spinasteryl-acetate) and mycosterols (ergosterol, ergosteryl acetate,  $\alpha$ -ergostenyl acetate), further bile acids, and derivatives (dehydrocholic acid, desoxycholic acid,  $\Delta^5$ -3-acetoxy-cholenic acid), and digitalis sapogenins (sarsasapogenin acetate, pseudo-sarsasapogenin acetate, diosgenin acetate, pseudo-diosgenin acetate). We included in Table I some steroids with hormonal properties and

TABLE II. COMPOUNDS WITH DEFINITE ANTICONVULSANT EFFECT

COMPOUND	DOSE IN MG. PER 100 GM. BODY WEIGHT	CONVULSION THRESHOLD AFTER INJECTION (A): THRESHOLD BEFORE INJECTION (B)
Testosterone (25 mg. per cubic centimeter sesame oil, HS)	17	1 to 1.2
Thirteen animals tested	18	1.5
	19	1 to 2
	21	1.7 only very mild convulsions
	25	2
	26	1.7 in large animal (169 Gm.)
Androstenedione (20 mg. per cubic centimeter olive oil, HS, St)	5	1.2 to 1.5
Twenty-seven animals tested	7	1.5 to >2.0
	8	>2
	10	1.0 to 3.0
	11	>3
	12	3 to >4
	15	2 to >4
	20	3
Dehydroandrosterone (20 mg. per cubic centimeter olive oil, HS, St)	8	1.1 to 2.5
Thirteen animals tested	10	1.8 to 2.5
	15	2.4
	20	1.6 to 3.0
	30	1.6 to 4.0
	40	2.4
Progesterone (12 mg. per cubic centimeter sesame oil, HS)	5	1.0
Ten animals tested	6.5	Ratio could not be determined exactly because only rudimentary twitchings were elicited
	8	Same
Acetoxypregnenolone (4 and 5 mg. per cubic centimeter sesame oil,* (HS)	2.0 and 2.5	1.0
Thirty-five animals tested	3	1.0 to 1.7
	4	2.1 to 3.7
	8	2.0 to 3.0
Desoxycorticosterone acetate (5 mg. per cubic centimeter cod-liver oil, HS)	20	1
Eight animals tested	23	1.2
	25	1 to 2.0
	27 and 28	>1.5; marked reduction of duration of convulsions
(15 mg. per cubic centimeter sesame oil, HS)	1 to 2	1.0 to 1.2
Eleven animals tested	2.5	1.7
	3.2	1.2 marked reduction of duration
	4.3	Unexcitable
	5	2.0 (duration reduced)
(15 mg. per cubic centimeter in male rats)		
Five animals tested		
(Weight, from 200 to 300 Gm.)	8 to 40	0.6 to 1
(Weight, from 150 to 170 Gm.)	35 to 48	No definite convulsions (current produced only brief single twitches)

\*Four milligrams per cubic centimeter prepared by us and 5 mg. per cubic centimeter prepared by Schering Corporation.

parent substances or derivatives that had no, or only a small, anticonvulsant effect in the doses at our disposal. This group comprises estrogenic hormones ( $\alpha$ -estradiol benzoate, theelin), the amorphous modification of 6( $\alpha$ ) acetoxyprogesterone, etiocholan-3 $\beta$ -ol-17-one acetate, 5-pregnen-3 $\beta$ -ol-20-one acetate, and 5, 16-pregnadien-3 $\beta$ -ol-20-one acetate. Finally, in Table I is contained stilbestrol that chemically does not belong in this group, but has folliculoid effects.

The steroids that manifested definite anticonvulsant effects as summarized in Table II belong to the groups of sex hormones and adrenal substances. It should be emphasized that as a rule anticonvulsant effects could be obtained with much smaller amounts, if high concentration rather than low concentrations of a compound were injected; for example, anticonvulsant effects were obtained with desoxycorticosterone acetate in a solution of low concentration (5 mg. per cubic centimeter) only if doses above 23 mg. per 100 Gm. body weight were injected, while from 2.5 to 3.2 mg. per 100 Gm. sufficed if a more concentrated solution (15 mg. per cubic centimeter) was used, apparently because an efficient blood concentration was more easily reached in the latter case.\* A further factor is the size (age) of the animal. In general, in rats weighing 100 Gm. or less, smaller relative doses (per 100 Gm. body weight) were sufficient to obtain an anticonvulsant effect than in rats weighing 150 Gm. or above. The sexual factor has already been mentioned. It is illustrated by the experiments with desoxycorticosterone acetate. In doses from 8 to 40 mg. per 100 Gm., this compound in concentrated solution produced in male rats weighing from 200 to 300 Gm. no change or even a slight increase of the convulsive reactivity; in smaller animals weighing from 150 to 170 Gm., a reduction of the reactivity was obtained with doses of from 35 to 48 mg.; this is about ten times the dose that was sufficient in female rats for such an effect.

In estimating the efficiency of an anticonvulsant substance, it seems desirable not only to determine its effect upon the convulsion threshold, but also upon the margin between anticonvulsant effect on the one hand and hypnotic effect on the other. In Table III these margins are shown for the steroids that we found to be distinctly anticonvulsant. In determining the minimum hypnotic dose, signs of depression of the activity of the central nervous system were looked for, such as reduction of spontaneous movements, ataxia, impairment of the righting reflexes, and reduction of the reactions to painful stimuli.

TABLE III. MARGIN BETWEEN MINIMUM ANTICONVULSANT AND HYPNOTIC EFFECT

COMPOUND	MINIMUM ANTICONVULSANT DOSE (MG.)	MINIMUM HYPNOTIC DOSE (MG.)
Testosterone	18 to 19	18 to 19
Androstenedione	7 to 10	10 to 12
Dehydroandrosterone	8 to 10	12 to 15
Progesterone	6.5	6.5
Acetoxypregnenolone	3 to 4	4 to 5
Desoxycorticosterone acetate		
5 mg. per cubic centimeter	25 to 26	25 to 26
15 mg. per cubic centimeter	2.5 to 3	2.5 to 3

\*It is, of course, of paramount importance to overcome the poor solubility of many of these if only for the sake of making the suspensions for several hours, or even slightly. Gently heating on a water bath resulted in no instances, for example, acetoxypregnenolone, to be in solution. In such a case the steroid was dissolved in a small amount of alcohol and the alcohol solution was mixed with the oil and the chloroform several days in a vacuum at room temperature. mixtures, without the steroid under study, were used; intraperitoneal injections of corresponding effective.



distinct rise in the base line. Bronchodilators, such as epinephrine, caused rather marked falls in the base line (Fig. 2). It is appreciated, of course, that our method will record only changes produced in the bronchi and not in the bronchioles or respiratory bronchioles. Since the bronchi act, in so far as is known, as do the smaller bronchioles, we feel the method to be valid.

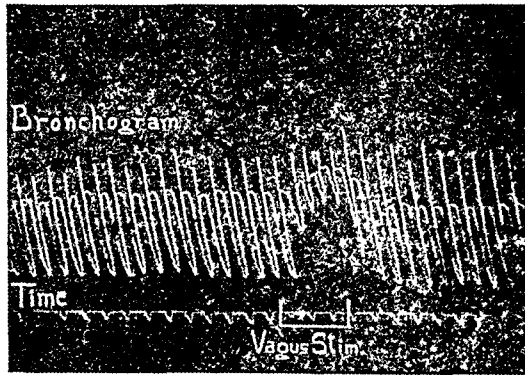


Fig. 1.—Effect of electrical stimulation upon the bronchogram.

TABLE I. EFFECT OF AGENTS OR PROCEDURES ON REFLEX BRONCHOCONSTRICTION INDUCED BY NASAL STIMULATION

	ATROPINE	ERGOTAMINE	VAGOTOMY	ATROPINE AND ERGOTAMINE
No effect	3	1	2	0
Effect diminished	2	2	3	0
Effect abolished	0	2	1	5

nature, the only other nervous mechanism capable of producing this change is a reduction in the tone of the sympathetic nerves innervating the bronchi. Very powerful bronchodilator nerves have been demonstrated by Dixon and Ransom,<sup>8</sup> so such a phenomenon is not unknown. Since ergotamine is capable of blocking sympathetic motor nerves,<sup>9</sup> then, with their activity abolished, a dependent reflex must likewise be abolished. We have found this to be true.

Before  
atropine

Bronchogram

Blood Pressure

Time

Chloroform

Fig. 3.

Fig. 3.—Effect of stimulation of the nasal mucous membrane by chloroform vapor before the use of atropine.

Fig. 4.—Effect of stimulation of the nasal mucous membrane by chloroform after 5 mg. of atropine.

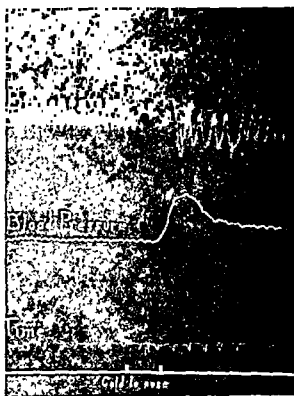
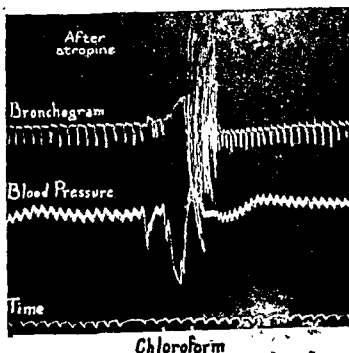


Fig. 4.



Chloroform

Fig. 5.—Effect of stimulation of the nasal mucous membrane by ethyl chloride after 10 mg. of atropine. Stimulation by ice water gives similar results.

If this work is valid and can be transposed to human subjects, then an interesting question is raised. For many years, the results of atropine therapy in the treatment of asthma have been very disappointing. In particular, atropine therapy of the asthmas resulting from exposure to cold air has been unsatisfactory. The explanation has been suggested that this is an allergy in which exposure to cold results in the local release of histamine, an extraordinarily powerful bronchoconstricting agent. It is possible that this mechanism might well be augmented by reflex constriction and, if this is a result of sympathetic inhibition, the ineffectiveness of atropine is understandable. Likewise, the efficacy of epinephrine and other sympathomimetic amines is understandable. It is well to keep this reflex in mind in breathing cold air through the nostrils at altitudes.

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## PROGRESS

### RH FACTORS IN CLINICAL MEDICINE\*

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ALMOST every important scientific discovery has a long historical background. Intimately associated with the history of the Rh blood factors is the name of the late Dr. Karl Landsteiner. Dr. Landsteiner is correctly known as the father of blood grouping, and in 1930 he was awarded the Nobel Prize in Medicine primarily for his discovery of human blood groups<sup>1, 2</sup> which made possible the safe transfusion of blood. Early in his studies Dr. Landsteiner conceived the existence of numerous serologic differences in human blood which might some day make possible the individual identification of human beings based on their blood reactions in a manner somewhat similar to the use of fingerprinting. The discovery of the subgroups in Groups A and AB by von Dungern and Hirsfeld<sup>3</sup> in 1910 and of the properties M, N, and P in 1928 by Landsteiner and Levine<sup>4</sup> increased the number of subdivisions of human blood from four to thirty-six; that was the status of the subject when I began my first studies on blood grouping in collaboration with Dr. S. H. Polayes in 1929. As a result of my interest in blood grouping, I became intimately acquainted with Dr. Landsteiner. Our first meetings soon blossomed into a close friendship which continued until his untimely death in 1943, depriving me of an irreplaceable friend and teacher and the medical world of one of its leading scientists.

Some of the earlier investigations on additional factors in human blood besides A, B, M, N, and P did yield some findings supporting Landsteiner's idea of the existence of numerous individual differences in human blood.<sup>5</sup> However, attempts to reproduce these findings were not consistently successful because no satisfactory method could be devised for obtaining the necessary antisera. In 1937 I became interested in the evolution of the agglutinogens M and N, and when my preliminary finding proved interesting,<sup>6</sup> I continued the work in collaboration with Dr. Landsteiner. In the blood of rhesus monkeys we detected the presence of M-like agglutinogens and also found that the injection of the blood of rhesus monkeys into rabbits stimulated the production of anti-M agglutinins.<sup>7</sup> Previously it had been found by Schiff that injections of sheep blood into rabbits caused the formation of anti-A agglutinins and hemolysins. It therefore occurred to us that by continuing this line of research, namely, the use of animal blood instead of human blood for preparing our antisera, we might discover new factors in human blood. Indeed, we did find<sup>8</sup> that some of our antisera for rhesus monkey blood contained an agglutinin reacting with the blood cells of 85 per cent of the white population independently of the blood groups or M, N, and P factors. The new factor detected by these rhesus anti-

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sera we later designated as Rh to indicate the manner in which it had first been discovered.

We did not immediately report our findings because we wished to perfect the method of producing the antisera. When, in 1939, Dr. H. Raymond Peters and I encountered three cases of hemolysis following transfusions of blood of the homologous group, which were proved to be due to isoimmunization to the rhesus factor,<sup>9</sup> it became evident that the new blood factor was of clinical importance. This is the reason why the Rh factor found by Landsteiner and myself in 1937 was first announced by us in January, 1940.<sup>8</sup>

As already mentioned, the blood cells of 85 per cent of all white individuals are agglutinated by the antirhesus sera. Such individuals are said to be Rh positive, while the remaining 15 per cent are said to be Rh negative. These two types of blood are hereditarily determined like the four blood groups, the Rh factor being transmitted as a single Mendelian dominant<sup>10</sup> by a pair of allelic genes, *Rh* and *rh*. Since every individual possesses a pair of genes from every series of allelic genes, one gene being derived from the mother and the other from the father, there are three genotypes possible. Rh-negative individuals belong to genotype *rrh* and are always homozygous; Rh-positive individuals may either be homozygous (genotype *RhRh*) or heterozygous (genotype *Rhrh*). Therefore, two Rh-negative parents can only have Rh-negative children. If one parent is Rh negative and the other is Rh positive, the children will all be Rh positive if the Rh-positive parent is homozygous, or one-half the children will be Rh positive and one-half Rh negative if the Rh-positive parent is heterozygous. When both parents are Rh positive, all the children will be Rh positive, except when the parents are both heterozygous, in which case one-fourth of the children will be Rh negative.<sup>11</sup>

Natural Rh isoantibodies have never been demonstrated in human serum. Therefore, Rh-negative individuals can safely be transfused with Rh-positive blood provided that they have not become sensitized to the Rh factor as a result of previous exposure to the Rh antigen. The situation with regard to Rh in relation to blood transfusion is therefore different from that which exists for the blood groups, in which every individual (except in infancy) has agglutinins in his or her serum for those agglutinogens (A, B or both) which are lacking from the erythrocytes, so that even a first transfusion of an incompatible blood may cause a hemolytic reaction. The situation is also different from that which exists for the properties M, N, and P. Natural antibodies for M and P have been demonstrated in a number of human sera<sup>12-14</sup> and in a few instances the transfusion of blood containing properties M and P has stimulated the production of anti-M and anti-P immune antibodies.<sup>15, 16, 17</sup> These instances are nevertheless extremely rare and no instance has yet been encountered of natural or immune isoagglutinins for N, so that it is evident that properties M, N, and P only rarely have to be considered when donors are selected for blood transfusion. The clinical importance of property Rh depends on the fact that Rh is a far better antigen in man than M, N, and P, as evidenced by the observation that as many as 90 per cent of cases of intra-group transfusion hemolysis can be traced to the Rh factor.<sup>18</sup>

Rh-negative individuals may become sensitized to Rh antigen in one of two ways: (1) sensitization may result from a transfusion of Rh-positive blood or (2) in women, sensitization may result from pregnancy with an Rh-positive fetus. Individuals sensitized by either of these two methods may have a serious hemolytic reaction if transfused with Rh-positive blood.<sup>9, 18</sup> Ideally,



therefore, every patient who is to be given a blood transfusion should be tested for the Rh factor as well as the blood group, and Rh-negative patients should only be given Rh-negative blood. Under battle conditions, of course, this precaution is hardly ever necessary because the casualties are mostly men who have never had a previous blood transfusion; in fact, it is undesirable because insistence on Rh tests would unduly delay a lifesaving procedure. In civilian hospitals such tests should be insisted on for all patients requiring a series of transfusions, and, if possible, for all women patients, particularly during the child-bearing age. It is important to mention at the onset that not every Rh-negative person receiving transfusions of Rh-positive blood or bearing an Rh-positive fetus becomes sensitized to the Rh factor. Individuals differ in the ease with which they can be sensitized, probably depending on some hereditary constitutional quality,<sup>19</sup> so that on the average only one in about twenty-five Rh-negative persons exposed to the Rh antigen becomes sensitized. This explains the relative infrequency of intragroup transfusion hemolysis due to the Rh factor, so that such cases were uncommon even before 1940 when the existence of the Rh factor was still unknown and tests for Rh were not carried out before transfusion.

Soon after the importance of the Rh factor as a cause of intragroup transfusion hemolysis was pointed out by Wiener and Peters, Levine and his collaborators<sup>20</sup> noticed that intragroup transfusion reactions occurred with exceptional frequency in women who had had stillbirths or infants with erythroblastosis fetalis. This suggested that isoimmunization in pregnancy might be the basis of the latter disease, which Diamond, Blackfan, and Baty<sup>21</sup> had previously shown to comprise a number of syndromes, namely, icterus gravis neonatorum, hemolytic anemia of the newborn, hydrops fetalis, and certain unexplained stillbirths. According to the theory of Levine and associates,<sup>22</sup> an Rh-negative woman bearing an Rh-positive fetus becomes sensitized to the Rh factor, the Rh antibodies produced by her pass through the placenta into the fetal blood circulation and combine with the red cells, giving rise to one or another manifestation of the disease. In support of this theory may be cited Levine's observation that 90 per cent of the erythroblastotic infants have mothers who are Rh negative, while, as already mentioned, only 15 per cent of individuals in the general population are Rh negative. Again, in about one-half of the cases, anti-Rh agglutinins could be demonstrated in the maternal serum; this proved that the mothers had been sensitized to the Rh factor. In view of this evidence concerning the nature of the disease, the name has been changed from erythroblastosis to hemolytic disease of the fetus and newborn or congenital hemolytic disease; the latter name will be used henceforth in this lecture.

While the mating of Rh-negative women with Rh-positive husbands occurs in one of ten marriages, congenital hemolytic disease affects only one of about 250 newborn infants. This suggests that only about one in twenty-five Rh-negative women becomes sensitized when bearing an Rh-positive fetus, just as in sensitization by blood transfusion. The firstborn is rarely affected, because it takes at least one pregnancy and sometimes more before a sufficient degree of sensitization develops. In most of the rare cases where the firstborn was affected, a careful history revealed that the woman had previously received a transfusion of Rh-positive blood.<sup>23, 24</sup> This serves to emphasize the importance of performing Rh tests before giving blood transfusions to women in the child-bearing age, and even to young girls, because such a transfusion could ruin a woman's chance to have a normal child.

While the theory of Levine and co-workers accounts for many of the essential facts in the pathogenesis of congenital hemolytic disease, a number of apparent paradoxes remain to be explained. Since the intact villus is impermeable to erythrocytes, it is not clear how the fetal Rh antigen gains access to the maternal circulation. There is no convincing evidence that Rh antigen exists in soluble form in body fluids,\* as is the case for the A and B factors, despite the assertions of certain workers.<sup>25, 25a</sup> The most plausible explanation is that during labor and delivery, villi become detached and enter the maternal circulation, carrying along enough fetal red cells to incite antibody formation by those women who are constitutionally predisposed. Autopsies on women who have died in childbirth or shortly thereafter have actually revealed the presence of Langerhans giant cells and villi in the lung bed. At the same time, this would explain why congenital hemolytic disease rarely affects the firstborn unless the mother has been previously sensitized by blood transfusion. It is difficult to reconcile Levine's theory with the lack of correlation between the titer of anti-Rh agglutinins in the maternal serum and the severity of the disease in the infants;<sup>26, 27</sup> thus in some of the most severe cases of congenital hemolytic disease no Rh agglutinins are detectable in the maternal serum; on the other hand, some Rh-negative women with anti-Rh agglutinins in their serum have given birth to apparently normal Rh-positive infants. The occasional occurrence of hemolytic disease when the mother is Rh positive has been attributed to subtypes of Rh and to the Hr factor, as will be explained later, and some cases have also been attributed to isoimmunization to the A-B blood group factors. With regard to the common blood groups, it is not clear why the A-B factors so rarely cause trouble, considering that the maternal serum frequently contains natural isoagglutinins anti-A and anti-B, and A and B are far more potent antigens than Rh. The suggestion that nonsecretory infants are the only ones affected is improbable *a priori* because as many as 15 per cent of all individuals are nonsecretors, while these cases are uncommon; besides, in two cases recently seen by me both infants proved to be secretors. Another puzzling feature of congenital hemolytic disease not explained by Levine's theory is why infants frequently appear perfectly normal when born, yet within a few hours or days severe jaundice and anemia develop.

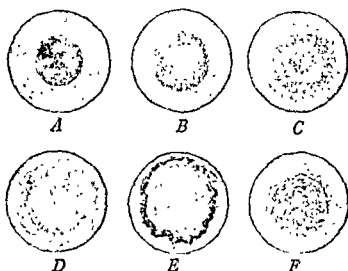
These paradoxes have been explained by the recent discovery<sup>28, 29</sup> that more than one variety of Rh antibody is produced by individuals sensitized to the Rh factor. In addition to anti-Rh agglutinins, antibodies designated "blocking antibodies" and "glutinins" are produced; these antibodies are far more important than the agglutinins in the causation of congenital hemolytic disease. The blocking antibodies and glutinins were not found previously because special techniques had to be devised for their detection, as will now be explained.

#### TESTS FOR RH SENSITIZATION

**Agglutination Test.**<sup>30</sup>—In the agglutination test for Rh sensitization, a drop of the patient's serum is mixed with a drop of a 2 per cent saline suspension of Group O Rh-positive cells in a small test tube and the mixture incubated in a water bath at body temperature for from thirty to sixty minutes. The reactions can usually be judged from the appearance of the sediment at the bottom of the tube (see Fig. 1). The sediment is then gently dislodged by twisting the tube and the mixture is examined for the presence or absence of

\*If the Rh antigens are actually present, their concentration must be far smaller than that of the A-B-O antigens.

agglutination. In positive reactions the red cells are clumped together in large masses usually visible to the naked eye, while in negative reactions the mixture remains homogeneous with each red cell separate and distinct, as proved by microscopic examination. When the patient's serum contains only very small



Magnification 1:2

Fig. 1.—Sedimentive method of testing for the Rh factor (magnification, 1:2). *a* and *b*, negative reactions; the inner light disc in *b*, is due to slight convexity in the bottom of the tube. *c*, Faintly positive reaction. *d*, Weak positive reaction. *e*, and *f*, Typical positive reactions. (From Landsteiner and Wiener: *J. Exper. Med.* 74: 312, 1941.)

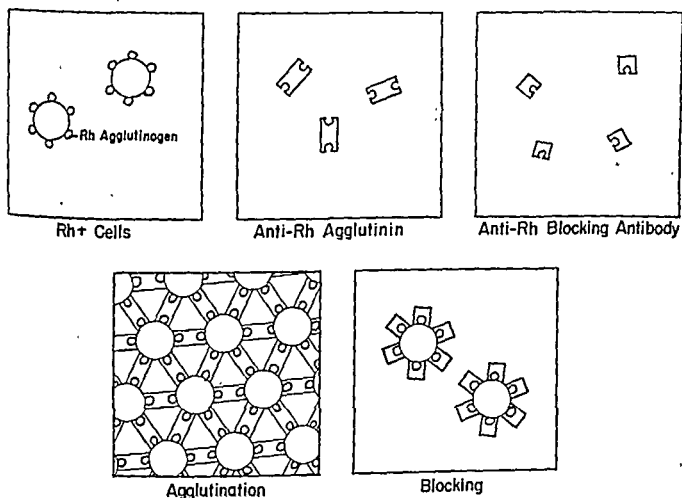


Fig. 2.—Diagrammatic representation of Rh agglutination and blocking reactions. (From Wiener, A. S.: *Ann. J. Clin. Path.* 15: 106, 1945.)

quantities of Rh agglutinin, centrifugation may be necessary to bring out the reaction. When enough agglutinin is present, on the other hand, strong clumping will occur even in tests with the patient's serum diluted with saline solution, and in tests on slides as well as in tubes.

According to our present concept, Rh agglutinins, like other antibodies in general, are modified serum globulins. Moreover, Rh agglutinins like other agglutinins are presumably polyvalent (probably bivalent) so that each anti-

body molecule has more than one specific combining group for the corresponding Rh hapten. Since the red cells presumably have numerous Rh haptens distributed over their surfaces, when agglutinin and agglutininogen combine, clumping results from the formation of a latticework, in accordance with Marrack's hypothesis (see Fig. 2). This<sup>31a, 31b</sup> concept of the nature of Rh agglutinins is useful in explaining the pathogenesis of congenital hemolytic disease, as will be pointed out later on.

*Blocking Test.*<sup>28, 32, 33</sup>—In search for an explanation of cases of congenital hemolytic disease which were typical except that the maternal serum contained no demonstrable Rh agglutinins, it occurred to me that Rh antibodies might be present but of a special variety incapable of bringing about agglutination. If such antibodies were present and combined with the Rh-positive red cells, then the cells might be expected to lose their capacity to agglutinate upon the further addition of anti-Rh agglutinating serum. In preliminary experiments carried out by me in 1941 some encouraging results were obtained, but final proof of the existence of such special antibodies, designated as Rh blocking antibodies, was not obtained until 1944 when larger quantities of potent human anti-Rh serum became available for these tests.<sup>28</sup>

In the test, a drop of the patient's serum is first mixed with a drop of a suspension of Group O Rh-positive red cells and incubated in the water bath. In the absence of Rh agglutinins, no clumping should be evident at this stage. Then a drop of a good human anti-Rh agglutinating serum is added, and the mixture is reincubated in the water bath. If the patient's serum does not contain any Rh antibodies, strong clumping should be evident; on the other hand, if the patient's serum contains Rh blocking antibodies, the red cells remain unagglutinated or the clumping is distinctly weakened.

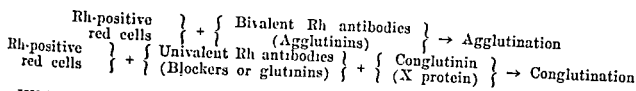
I have postulated that Rh blocking antibodies, in contrast to Rh agglutinins, are univalent.<sup>28</sup> This would account for the ability of these antibodies to combine with Rh agglutininogen and to block the combining sites on the surface of the erythrocytes, but without clumping them (see Fig. 2). If this concept is correct, it seems reasonable to conclude that Rh blocking antibodies are comprised of smaller molecules than Rh agglutinins. Therefore, Rh blocking antibodies should be capable of traversing the placental barrier into the fetal circulation more readily and earlier in pregnancy than Rh agglutinins. This is supported by the clinical observation that the presence of Rh blocking antibodies is of more serious prognostic import than the presence of Rh agglutinins without blocking antibodies; for example, when blocking antibodies are present, stillbirths are more likely to result.

The discovery of the Rh blocking antibodies has also served to explain certain intragroup hemolytic transfusion reactions. For example, I have encountered many cases of severe transfusion hemolysis in male patients due to Rh isoimmunization by repeated transfusions. In two such cases, the patients were Rh negative, but their sera contained no anti-Rh agglutinins, and in the usual compatibility test the sera of the patients failed to agglutinate the blood suspensions of the donors whose blood caused the reactions. However, the blocking tests were positive in both cases and showed the presence of Rh antibodies of high titer.

*Conglutination Test.*<sup>29</sup>—The discovery of the Rh blocking antibodies explains why the classic cross-matching tests have been inadequate to detect all cases of intragroup incompatibility. By slightly modifying the technique of the compatibility test, it is now possible to avoid this pitfall.

The new test for Rh sensitization, designated as the conglutination test, differs from the agglutination test in that it occurs in two stages instead of one and because a third component besides the Rh antigen and its specific antibody must be present in the mixture in order for clumping to occur. The third component, like complement in serologic hemolysis, is absorbed onto the specifically sensitized red cells and then causes them to stick together. However, this third component is distinct from complement. It resists heating at 60° C. and is a colloidal constituent in the plasma probably identical with the so-called X protein, which is a large molecular complex of albumin, globulin, and phospholipid. The Rh antibody participating in the conglutination reaction is univalent and is best referred to as Rh glutinin, with the understanding that it is still uncertain whether or not glutinin is identical with blocking antibody.

The difference between Rh agglutination and Rh conglutination can be summarized as follows:



While X protein is heat stable, it has the peculiarity that it dissociates readily into its constituent smaller molecules of albumin and globulin upon slight dilution of the plasma with water. This explains why glutinins do not give rise to clumping under the conditions existing in the agglutination test, because the use of saline in preparing the red blood cell suspensions and for diluting the patient's serum is sufficient to dissociate partly or completely the X protein. The conglutination test is therefore carried out like the agglutination test except that the use of saline or any crystalline solution is strictly avoided. The red cells are suspended in the individual's own plasma or serum or more conveniently in inactivated Group AB serum. The patient's serum is tested undiluted, or in titrations the serum is diluted with Group AB serum instead of saline solution. I believe that the slide test of Diamond and Abelson,<sup>34, 35</sup> and the capillary tube test of Chown,<sup>36</sup> when properly carried out, depend for their success on a conglutination reaction rather than on an agglutination reaction, as originally stated by these workers. Since most Rh antisera will contain both univalent and bivalent antibodies, then, depending on the conditions of the test, one will obtain agglutination or conglutination or both.

In tests for Rh sensitization by the conglutination technique, positive reactions will be obtained in all cases in which the patient's serum contains Rh agglutinins or Rh blocking antibodies, or both, and occasionally when the other two tests are negative or inconclusive.<sup>29, 35</sup> The conglutination test is the most reliable indication of the presence or absence of Rh sensitization because it is always or almost always positive if the patient is sensitized. Moreover, glutinin is univalent and therefore presumably comprised of small molecules, so this antibody, like blocking antibody, plays a more important role than Rh agglutinin in the pathogenesis of congenital hemolytic disease.<sup>19</sup>

The bizarre behavior of congenital hemolytic disease, so that some infants do not present signs of hemolysis until several hours or days after birth, can probably be attributed to X protein, the third component necessary for *in vitro* conglutination, and therefore probably important for *in vivo* hemolysis.<sup>29</sup> Perhaps in these cases X protein does not form until after birth, at which time the profound physiologic changes occurring bring about aggregation of serum proteins into more complex molecules. In this connection Pedersen<sup>37</sup> has found

that serum from cow's fetus and newborn calves and foals contains large amounts of a special globulin of low molecular weight, designated fetuin by him, which he believes to be one of the precursors of X protein. Fetuin has also been found in human umbilical cord serum, although in smaller amount.

### Rh BLOOD TYPES

Before proceeding with the presentation of illustrative clinical material, a brief description of the Rh blood types is in order.

While our antirhesus guinea pig and rabbit sera all gave parallel reactions,<sup>10</sup> agglutinating the blood of approximately 85 per cent of all white individuals, it was soon found that human antisera from Rh-negative patients sensitized to the Rh factor varied in their specificities. The great majority of the human antisera gave reactions identical with those of the antirhesus sera; such human antisera are now designated as standard anti-Rh or anti-Rh<sub>0</sub>.<sup>39</sup> The variations among human antisera were soon explained by the demonstration of two additional varieties of Rh agglutinins, one giving 70 per cent positive reactions<sup>18, 38</sup> and designated<sup>39, 40</sup> as anti-Rh', the other giving 30 per cent positive reactions<sup>41, 42</sup> and designated anti-Rh''. If blood specimens are tested with all three antisera, anti-Rh<sub>0</sub>, anti-Rh', and anti-Rh'', eight distinct types of human blood can be identified instead of the two, Rh positive and Rh negative, detected with the aid of standard human anti-Rh<sub>0</sub> serum or animal antirhesus serum alone. As I<sup>43</sup> have shown, the three Rh antisera detect three corresponding Rh factors, Rh<sub>0</sub>, Rh' and Rh'', which in combination give rise to at least five Rh agglutinogens instead of one, namely, Rh<sub>0</sub>, Rh', Rh'', Rh<sub>1</sub> (or Rh'<sub>0</sub>), and Rh<sub>2</sub> (or Rh''<sub>0</sub>). These five agglutinogens in combination determine the eight Rh types, which are hereditarily transmitted by means of at least six allelic genes,<sup>44</sup> Rh<sub>0</sub>, Rh' Rh'', Rh<sub>1</sub>, Rh<sub>2</sub>, and rh.

Anyone familiar with the serology and genetics of the four Landsteiner blood groups can readily master the eight Rh blood types. First, considering only the reactions of anti-Rh' and anti-Rh'', these two antisera determine four types of blood entirely analogous serologically and genetically to the four blood groups. If, in addition, the reactions with anti-Rh<sub>0</sub> serum are taken into account, eight types of blood result, as shown in Table I. The types are named

TABLE I. THE EIGHT RH BLOOD TYPES\*

BLOODS CONTAINING Rh <sub>0</sub>				BLOODS LACKING Rh <sub>0</sub>			
DESIGNATIONS OF TYPES	REACTIONS WITH SERA			DESIGNATIONS OF TYPES	REACTIONS WITH SERA		
	anti-Rh'	anti-Rh''	anti-Rh <sub>0</sub>		anti-Rh'	anti-Rh''	anti-Rh <sub>0</sub>
Rh <sub>0</sub>	-	-	+	Rh-	-	-	-
Rh <sub>1</sub> (Rh' <sub>0</sub> )	+	-	+	Rh'	+	-	-
Rh <sub>2</sub> (Rh'' <sub>0</sub> )	-	+	+	Rh''	-	+	-
Rh <sub>1</sub> Rh <sub>2</sub> (Rh' <sub>0</sub> Rh'' <sub>0</sub> )	+	+	+	Rh'Rh''	+	+	-

\*Wiener, A. S.: Am. J. Clin. Path. 15: 106, 1945.

after the antisera with which they react.<sup>39, 40</sup> For example, type Rh<sub>0</sub> reacts with anti-Rh<sub>0</sub> but not with anti-Rh' or anti-Rh''; similarly, type Rh' reacts with anti-Rh' but not with anti-Rh<sub>0</sub> or anti-Rh''; etc. Blood reacting with anti-Rh' and anti-Rh<sub>0</sub> but not with anti-Rh'' is designated Rh<sub>1</sub> (short for Rh'<sub>0</sub>) instead of Rh<sub>0</sub>Rh' because the factors Rh<sub>0</sub> and Rh' in such blood are ordinarily hereditarily transmitted as a unit, like the agglutinogens A<sub>1</sub> and A in blood of Sub-group A<sub>1</sub> rather than like the agglutinogens A and B in Group AB blood. Thus, in the mating Rh negative × Rh<sub>1</sub>, as a rule, either the children are all type Rh<sub>1</sub>, or

one-half belong to type  $Rh_1$  while the other one-half are Rh negative. To be sure, the factors  $Rh_0$  and  $Rh'$  will segregate in occasional matings involving type  $Rh_1$  individuals, namely, when type  $Rh_1$  individual belongs to genotype  $Rh_0Rh'$ , but such cases are rare and have not yet been encountered because of the low frequencies of genes  $Rh_0$  and  $Rh'$ . For the same reason, blood reacting with anti- $Rh''$  and anti- $Rh_0$  but not with anti- $Rh'$  is designated as type  $Rh_2$  (or  $Rh_0''$ ) rather than  $Rh_0Rh''$ . On the other hand, types  $Rh'Rh''$  and  $Rh_1Rh_2$  are so designated because, with rare exceptions, such bloods contain two separate agglutinogens.

The Rh blood types, like the blood groups and M-N types, exhibit striking differences in distribution in various races<sup>44</sup> and promise to have important applications in anthropology. Some of the findings on the racial distribution of the Rh blood types are summarized in Table II. Chinese,<sup>45, 46</sup> Japanese,<sup>47, 48</sup>

TABLE II. DISTRIBUTION OF THE RH BLOOD TYPES

POPULATION TESTS	NUMBER OF PERSONS TESTED	FREQUENCIES OF Rh TYPES (PER CENT)							
		Rh-	Rh <sub>1</sub>	Rh <sub>2</sub>	[Rh <sub>1</sub> Rh <sub>2</sub> ]	Rh <sub>0</sub>	Rh'	Rh''	[Rh'Rh'']
Caucasians (New York City) { a*	1000	12.9	54.1	12.8	16.4	2.6	0.9	0.3	0
b†	818	13.5	55.6	15.8	12.0	1.7	1.1	0.3	0
c‡	2438	14.5	52.5	15.7	13.1	2.4	1.1	0.7	0.02
Caucasians (England)§§§	927	14.8	54.9	12.2	13.7	2.5	0.7	1.3	0
Caucasians (Australia)§	350	14.9	54.0	12.6	16.5	0.6	0.9	0.5	0
Negroes (New York City)**	223	8.1	20.2	22.4	5.4	41.2	2.7	0	0
Asiatic Indians††	156	7.1	70.5	5.1	12.8	1.9	2.6	0	0
Chinese‡‡	132	1.5	60.6	3.0	34.1	0.8	0	0	0
Mexican Indians§§	98	0	48.0	9.2	41.8	1.0	0	0	0
Japanese { a***	150	1.3	37.4	13.3	47.3	0	0	0	0.7
b†††	180	0.6	51.7	8.3	39.4	0	0	0	0
Filipinos§	100	0	87.0	2.0	11.0	0	0	0	0
Indonesians§	100	0	75	2	22	0	0	0	1

\*Old Series; Wiener, A. S.: Am. J. Clin. Path. 15: 106, 1945.

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‡Unger, L. J.: Personal Communication.

§Simmons, R. T.: Personal Communication.

\*\*Wiener, Belkin, and Sonn.<sup>53</sup>

††Wiener, Sonn, and Belkin: J. Immunol. 50: 341, 1945.

‡‡Wiener, Sonn, and Yi.<sup>46</sup>

§§Wiener, Zepeda, Sonn, and Polivka.<sup>50</sup>

\*\*\*Waller and Levine.<sup>47</sup>

†††Miller and Taguchi.<sup>48</sup>

§§§Race, R. R.: Personal communication.

American Indians,<sup>49, 50</sup> Filipinos, Hawaiians,<sup>51</sup> and Australian aborigines<sup>52</sup> are characterized by the virtual absence of the Rh-negative type; in Negroes<sup>53, 57</sup> the most striking finding is the high incidence of type  $Rh_0$ , which is about twenty times as frequent as in the white race. Some correlation between the frequency of the Rh-negative type in the population and the incidence of congenital hemolytic disease is to be expected, and it is of interest to note that Levine and Wong<sup>45</sup> report that this disease is virtually unknown among Chinese.

The theory of six allelic genes has been tested by studying the Rh blood types in families<sup>54-56</sup> and by the gene frequency method; the findings support the theory in its essential details. In Table III are presented our findings on the heredity of the Rh blood types in 197 families with 463 children. Only two apparent contradictions to the genetic theory were encountered and both of these proved to be due to illegitimacy. In the statistical test by the gene frequency method, the findings on white individuals<sup>57, 58</sup> have been in good agreement with the theory of six allelic genes. In American Indians and Mongolians, however,

TABLE III. SUMMARY OF AUTHOR'S FAMILY STUDIES ON THE RH BLOOD TYPES\*

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES							
		Rh-	Rh <sub>1</sub>	Rh <sub>2</sub>	Rh <sub>1</sub> Rh <sub>2</sub>	Rh <sub>0</sub>	Rh'	Rh''	TOTALS
Neg. × Neg.	4	14	0	0	0	0	0	0	14
Neg. × Rh <sub>1</sub>	49	25	73	0	0	7	0	0	105
Neg. × Rh <sub>2</sub>	16	10	0	20	0	0	0	0	30
Neg. × Rh <sub>1</sub> Rh <sub>2</sub>	15	(1)	18	14	0	0	0	0	33
Neg. × Rh <sub>0</sub>	3	3	0	0	0	3	0	0	6
Neg. × Rh'	2	1	0	(1)	0	0	1	0	3
Neg. × Rh''	1	1	0	0	0	0	0	5	6
Rh <sub>1</sub> × Rh <sub>1</sub>	26	5	62	0	0	4	1	0	72
Rh <sub>1</sub> × Rh <sub>2</sub>	21	6	15	7	18	0	2	0	48
Rh <sub>1</sub> × Rh <sub>1</sub> Rh <sub>2</sub>	27	0	46	8	25	0	0	0	79
Rh <sub>1</sub> × Rh <sub>0</sub>	1	1	0	0	0	0	0	0	1
Rh <sub>1</sub> × Rh'	3	0	3	0	0	0	0	0	3
Rh <sub>1</sub> × Rh''	4	1	2	0	3	0	0	0	6
Rh <sub>2</sub> × Rh <sub>2</sub>	2	1	0	4	0	0	0	0	5
Rh <sub>2</sub> × Rh <sub>1</sub> Rh <sub>2</sub>	6	0	2	8	8	0	0	0	18
Rh <sub>2</sub> × Rh <sub>0</sub>	2	0	0	1	0	4	0	0	5
Rh <sub>1</sub> Rh <sub>2</sub> × Rh <sub>1</sub> Rh <sub>2</sub>	10	0	3	1	17	0	0	0	21
Rh <sub>1</sub> Rh <sub>2</sub> × Rh <sub>0</sub>	2	0	1	1	0	0	0	0	2
Rh <sub>1</sub> Rh <sub>2</sub> × Rh'	2	0	1	1	2	0	0	0	4
Rh <sub>0</sub> × Rh'	1	0	0	0	0	1	1	0	2
Totals	197	69	226	66	73	19	5	5	463

\*Wiener and Sonn.<sup>50</sup>

the calculated sum of the gene frequencies falls short of 100 per cent, and this we<sup>50</sup> attribute to the occurrence in these peoples of an exceptionally high frequency of the so-called genes *Rh<sub>1</sub>* and/or *Rh<sub>2</sub>* of Race and Taylor.<sup>50, 60</sup> Negroes are characterized by an exceptionally high percentage of individuals with blood giving weak or intermediate reactions with one or more of the Rh antisera,<sup>57</sup> and these have been attributed to the existence of still more genes in the Rh allelic genes,<sup>61</sup> the "intermediate genes." For practical purposes, the theory of six allelic genes has proved adequate, because the genes *Rh<sub>1</sub>* and/or *Rh<sub>2</sub>* and the intermediate genes are quite rare among white individuals. Under the theory of six allelic genes, twenty-one genotypes are possible, and the genotypes corresponding to each of the eight Rh blood types are readily ascertained, as shown in Table IV. If the genes *Rh<sub>1</sub>* and/or *Rh<sub>2</sub>* and the intermediate genes are taken into account, the situation naturally becomes far more complicated.

TABLE IV. THE EIGHT RH TYPES AND THEIR GENOTYPES

Rh TYPES	GENOTYPES
Neg.	<i>rh rh</i>
Rh'	<i>Rh'Rh'</i> and <i>Rh'rh</i>
Rh''	<i>Rh''Rh''</i> and <i>Rh''rh</i>
Rh'Rh''	<i>Rh'Rh''</i>
Rh <sub>0</sub>	<i>Rh<sub>0</sub>Rh<sub>0</sub></i> and <i>Rh<sub>0</sub>rh</i>
Rh <sub>1</sub> (Rh <sub>0</sub> ')	<i>Rh<sub>1</sub>Rh<sub>1</sub></i> , <i>Rh<sub>1</sub>rh</i> , <i>Rh<sub>1</sub>Rh'</i> , <i>Rh<sub>1</sub>Rh<sub>2</sub></i> , and <i>Rh'Rh<sub>1</sub></i>
Rh <sub>2</sub> (Rh <sub>0</sub> '')	<i>Rh<sub>2</sub>Rh<sub>2</sub></i> , <i>Rh<sub>2</sub>rh</i> , <i>Rh<sub>2</sub>Rh''</i> , <i>Rh<sub>2</sub>Rh<sub>0</sub></i> , and <i>Rh''Rh<sub>2</sub></i>
Rh <sub>1</sub> Rh <sub>2</sub> (Rh <sub>0</sub> 'Rh <sub>0</sub> '')	<i>Rh<sub>1</sub>Rh<sub>2</sub></i> , <i>Rh<sub>1</sub>Rh''</i> , and <i>Rh'Rh<sub>2</sub></i>

## THE HR FACTOR

The literature on the Hr factor is somewhat confused, because the early work was done with weak antisera, so that some of the findings were inaccurate and misleading. Levine and Javert<sup>62</sup> first reported that they had detected in the serum of an Rh-positive mother of an erythroblastotic infant an antibody which had the property of agglutinating all Rh-negative bloods. Because of this property of the serum, the new agglutinable property detected by it was designated as Hr and the corresponding agglutinin as anti-Hr. Levine reported that his anti-Hr serum gave about 30 per cent positive reactions and that all Rh-



positive bloods not agglutinated by anti-Rh' reacted with anti-Hr. On this basis he postulated that Hr and Rh were allelic, without further clarifying his hypothesis. He also asserted that Hr incompatibility must be considered as a possibility whenever the Rh-positive mother has an Rh-negative child<sup>63</sup> and that all type Rh<sub>1</sub>Rh<sub>2</sub> individuals are Hr negative.<sup>47</sup> As will be shown, the latter two statements have been contradicted by subsequent findings.

In 1943, Race and Taylor<sup>64</sup> also detected in the serum of an Rh-positive mother of an erythroblastotic infant an agglutinin which reacted with all Rh-negative bloods but differed from Levine and Javert's findings in that it gave 80 per cent positive reactions. As has been pointed out by Wiener and co-workers,<sup>65</sup> however, Race and Taylor's serum detected the same blood property as Levine and Javert's serum, the lower percentage of positive reactions obtained by Levine being due to the use of a weaker antiserum which missed bloods heterozygous for the Hr factor. In this lecture, therefore, the blood property studied by the British investigators will also be designated as Hr. As postulated by Race and associates,<sup>66</sup> Hr is a blood factor present in the agglutinogens determined by genes Rh<sub>1</sub>, Rh'', Rh<sub>0</sub>, and rh but absent from the agglutinogens determined by genes Rh<sub>1</sub> and Rh'. From the reactions determined by the six genes (Table V), the reactions, of the twenty-one genotypes are readily ascer-

TABLE V. THE REACTIONS DETERMINED BY THE SIX STANDARD GENES AND GENES Rh<sub>1</sub> AND Rh<sub>2</sub>

GENES	REACTIONS WITH Rh SERA			REACTIONS WITH Hr ANTISERUM
	anti-Rh'	anti-Rh''	anti-Rh <sub>0</sub>	
rh	Neg.	Neg.	Neg.	Pos.
Rh <sub>0</sub>	Neg.	Neg.	Pos.	Pos.
Rh'	Pos.	Neg.	Neg.	Neg.
Rh <sub>1</sub>	Pos.	Neg.	Pos.	Neg.
Rh''	Neg.	Pos.	Neg.	Pos.
Rh <sub>2</sub>	Neg.	Pos.	Pos.	Pos.
Rh <sub>1</sub>	Pos.	Pos.	Neg.	Neg.
Rh <sub>2</sub>	Pos.	Pos.	Pos.	Neg.

tained, as shown in Table VI. In giving the expected reactions with anti-Hr serum, it is postulated that a single dose of a gene positive for Hr determines a weaker reaction with anti-Hr than a double dose of Hr-positive genes. (This idea is supported by observations made in tests with weak anti-Hr serum,<sup>64, 65</sup> while in tests with potent Hr antisera it is difficult or impossible to demonstrate the gene-dose effect.) It will be seen that the expected percentage of positive reactions, approximately 80 per cent, closely agree with that reported by Race and Taylor and that observed by me. If only the strong reactions are counted, then the percentage of positives becomes 30 per cent, as reported by Levine. This explains the apparent contradiction between the report of Levine, and Race and Taylor, and why Levine thought that type Rh<sub>1</sub>Rh<sub>2</sub> bloods were Hr negative. Since Hr-negative mothers can only belong to genotype Rh<sub>1</sub>Rh<sub>2</sub>, Rh<sub>1</sub>Rh', or Rh'Rh', they must transmit either an Rh<sub>1</sub> or an Rh' gene to every child and so cannot have any Rh-negative children, contrary to the assertion made by Levine.

The main clinical application of the Hr factor is as a presumptive test for homo- or heterozygosity of type Rh<sub>1</sub> fathers in families with erythroblastotic infants.<sup>64</sup> The diagnosis is only *presumptive* because there are five genotypes in type Rh<sub>1</sub>, while anti-Hr serum merely subdivides type Rh<sub>1</sub> into two parts. Hr-negative individuals of type Rh<sub>1</sub> are almost surely homozygous for Rh<sub>1</sub>, while

TABLE VI. RELATION OF THE HR FACTOR TO THE RH BLOOD TYPES

Rh BLOOD TYPE	REACTIONS WITH Rh SERA			GENOTYPES	REACTIONS WITH Hr ANTI-SERUM*	PER CENT OBSERVED (350 TESTS ON WHITE INDIVIDUALS, N.Y.C.)
	anti-Rh'	anti-Rh''	anti-Rh <sub>0</sub>			
Rh-	Neg.	Neg.	Neg.	<i>rrrh</i>	Strong	12.3
Rh'	Pos.	Neg.	Neg.	$\left\{ \begin{array}{l} R'Rh' \\ Rh'rh \end{array} \right\}$	Neg. Weak	0 1.1
Rh''	Neg.	Pos.	Neg.	$\left\{ \begin{array}{l} R''R'' \\ R''rh \end{array} \right\}$	Strong	0.9
Rh'Rh''†	Pos.	Pos.	Neg.	<i>Rh'Rh''</i>	Weak	0
Rh <sub>0</sub>	Neg.	Neg.	Pos.	$\left\{ \begin{array}{l} Rh_0Rh_0 \\ Rh_0rh \end{array} \right\}$	Strong	1.1
Rh <sub>1</sub>	Pos.	Neg.	Pos.	$\left\{ \begin{array}{l} Rh_1Rh_1 \\ Rh_1Rh' \\ Rh_1rh \end{array} \right\}$	Neg.	19.7
				$\left\{ \begin{array}{l} Rh_1Rh_0 \\ Rh_1Rh'' \end{array} \right\}$	Weak	36.9
Rh <sub>2</sub>	Neg.	Pos.	Pos.	$\left\{ \begin{array}{l} Rh_2Rh_1 \\ Rh_2Rh'' \end{array} \right\}$	Strong	16.0†
				$\left\{ \begin{array}{l} Rh_2rh \\ Rh_2Rh_0 \\ Rh''Rh_0 \end{array} \right\}$		
Rh <sub>1</sub> Rh <sub>2</sub> ‡	Pos.	Pos.	Pos.	$\left\{ \begin{array}{l} Rh_1Rh_1 \\ Rh_1Rh'' \end{array} \right\}$	Weak	11.7
				$\left\{ \begin{array}{l} Rh_1Rh_0 \\ Rh_1Rh_2 \end{array} \right\}$	Neg.	0.3

\*Theoretically expected reactions. Actually the Hr antiserum used was of such high titer that all positive bloods gave uniformly strong reactions.

†Includes one blood giving intermediate reactions (Rh<sub>1</sub>).

‡If the existence of both genes *Rh<sub>1</sub>* and *Rh<sub>2</sub>* is admitted, then there are nineteen genotypes possible in these types instead of four as given in Table IV. The additional fifteen genotypes, if they actually exist, must all be quite rare, so for simplicity only the most common, genotype *Rh<sub>1</sub>Rh<sub>2</sub>*, is given in this table.

Hr-positive individuals of type Rh<sub>1</sub> are almost always heterozygous and bear gene *rh*.

The accuracy of the theory of Race and co-workers regarding the Hr factor has been confirmed by family studies and by the gene frequency method.<sup>65, 67</sup> Since every gene negative for factor Rh' is positive for factor Hr, it is evident that factors Rh' and Hr are related to each other serologically and genetically like M and N (see Wiener and co-workers,<sup>65</sup> Levine<sup>69</sup>). To indicate this (especially since Fisher<sup>70</sup> has postulated the existence of three factors in Rh-negative blood corresponding to the three Rh factors), the standard Hr factor which we have described is perhaps better known as Hr' and the corresponding serum as anti-Hr'. Of the other two Hr factors postulated by Fisher, Hr'' has recently been found by Mourant,<sup>71</sup> while convincing evidence is still lacking concerning the existence of Hr<sub>0</sub>.<sup>68</sup>

#### CLINICAL APPLICATION

A few cases will now be described which illustrate the application of the Rh blood types in relation to diagnosis, treatment, and prognosis of congenital hemolytic disease.

CASE 1.—I was requested by Dr. J. Hoffman, at the Israel Zion Hospital, to see a deeply jaundiced infant, 2 months of age, in order to determine whether he was suffering from congenital stricture of bile ducts or congenital hemolytic disease.

The mother of the infant gave the following obstetric history: The first pregnancy terminated at term, in 1935, with a normal female child. The second pregnancy, in 1939,

yielded a male infant who developed anemia after birth but recovered following transfusion therapy. The third pregnancy, in 1945, resulted in the patient, who shortly after birth developed jaundice and anemia for which he was treated by transfusions of Rh-negative blood. The anemia improved, but deep jaundice persisted.

The infant's mother had never received a blood transfusion.

The grouping and Rh tests gave the results listed in Table VII.

The fact that the mother was Rh negative, while the father and all three children were Rh positive could, of course, be merely coincidental. However, in the maternal serum anti-Rh, agglutinins of titer 16 were detected, demonstrating that she was sensitized to the Rh factor and establishing the diagnosis of congenital hemolytic disease.

TABLE VII

BLOOD OF	GROUPS AND SUBGROUPS	MN TYPE	Rh TYPE	Hr REACTION
Father	O	M	Rh <sub>1</sub>	Pos.
Mother	A <sub>1</sub>	MN	Neg.	Pos.
Ten-year-old sister	O	MN	Rh <sub>1</sub>	Pos.
Six-year-old brother	A <sub>1</sub>	MN	Rh <sub>0</sub>	Pos.
Patient	O	MN	Rh <sub>1</sub>	Pos.

The case is typical in that it demonstrates how the first Rh-positive infant escapes the disease, while the subsequent infants are affected progressively more severely as the sensitization of the mother increases in degree with each pregnancy. The second infant recovered fully even although the donors whose blood was used for the transfusions were not selected with regard to the Rh factor, then unknown. The patient was treated properly with transfusions of Rh-negative blood and nevertheless had an unusual complication in that damage to the liver finally caused death at the age of 3 months.

The Hr factor is of no assistance in determining hetero- or homozygosity of the father in this case, because he does not belong to type Rh<sub>1</sub> but to type Rh<sub>2</sub>. The fact that he has had children of types Rh<sub>1</sub> and Rh<sub>0</sub> proves that he belongs to genotype Rh<sub>2</sub>Rh<sub>0</sub>. Therefore, every future child of this couple must be Rh positive, either type Rh<sub>1</sub> or Rh<sub>0</sub>, and would be certain to have congenital hemolytic disease.

CASE 2.—This patient was referred by Dr. B. Meriam with the history that she was in the eighth month of pregnancy and that the fetal heart sounds had not been audible for the past three weeks. This was the patient's third pregnancy. The first pregnancy had terminated with a normal female child 4 years old at this time, while the second pregnancy had yielded a seven-month macerated fetus.

Tests on the patient, her husband, and daughter showed the results listed in Table VIII.

TABLE VIII

BLOOD OF	GROUP AND SUBGROUP	MN TYPE	Rh TYPE	Hr REACTION
Husband	O	MN	Rh <sub>1</sub>	Neg.
Patient	A <sub>1</sub>	M	Neg.	Pos.
Daughter	A <sub>1</sub>	M	Rh <sub>1</sub>	Pos.

No Rh agglutinins were demonstrable in the patient's serum, but Rh blocking antibodies were present in low titer (2 units). By the conglutination technique, Rh antibodies were readily demonstrable in the patient's serum in dilutions as high as 1 to 50. These findings therefore established the diagnosis of congenital hemolytic disease as the cause of the stillbirth after the second pregnancy and the intrauterine death during the present pregnancy.

Shortly after these tests were carried out the patient gave birth to a macerated fetus, after which she had a hemorrhage which was treated by plasma transfusions. The patient probably owes her life to the newer knowledge of the Rh blood factors and the blocking antibodies. Under similar circumstances before the discovery of the Rh factor, the patient

would have been treated by transfusions of whole blood from an unselected and therefore probably Rh-positive, Group A donor, especially since blocking antibodies are not detectable in the usual cross-matching tests.

The more severe manifestations of congenital hemolytic disease in this case, as contrasted with Case 1, is in line with the remarks concerning the more serious prognostic significance of Rh blocking antibodies as compared with Rh agglutinins. Moreover, the husband of the patient is Hr negative so that the prognosis for further pregnancies for this couple is practically hopeless, because every future pregnancy would be expected to yield an Rh-positive fetus and therefore terminate with another stillbirth. In Case 1, on the other hand, where the degree of Rh sensitization of the mother is not as pronounced, future children, although certain to be affected with congenital hemolytic disease, might nevertheless prove viable and recover after transfusion therapy.

CASE 3.—This case was referred to me by Dr. I. Frohman, who is reporting it in detail elsewhere.<sup>72</sup> The patient was first admitted to the Rockaway Beach Hospital during the sixth month of her eleventh pregnancy because of an abruptio placentae. She was delivered shortly afterward of a stillborn fetus with hydrops.

The obstetric history and the results of grouping and Rh tests are given in Tables IX and X.

No anti-Rh agglutinins were demonstrable in the patient's serum, but Rh blocking antibodies were present in low titer (2 units). These findings therefore indicate that the stillbirths that this patient had after the fifth pregnancy were due to congenital hemolytic disease. The stillbirth occurring after the third pregnancy must be attributed to some mechanism other than isoimmunization in pregnancy.

TABLE IX

BLOOD OF	GROUP	MN TYPE	Rh TYPE
Husband	O	MN	Rh <sub>2</sub>
Patients	O	N	Neg.

TABLE X

PREGNANCIES	GROUP	MN TYPE	Rh TYPE
1. 1928—Normal male infant	O	MN	Rh <sub>2</sub>
2. 1929—Normal male infant	O	MN	Rh <sub>2</sub>
3. 1930—Premature stillbirth at 6½ months; Patient had hypertension during pregnancy			
4. 1932—Normal male infant	O	N	Neg.
5. 1935—Female infant; jaundice on eighth day; cleared in a few days	O	N	Rh <sub>2</sub>
6. 1936—Normal male infant	O	MN	Neg.
7. 1938—Stillborn male			
8. 1938—Fraternal twins in separate sacs; male twin normal; female twin died after thirteen hours from icterus gravis and hydrops	O	MN	Neg.
9. 1940—Normal male infant	O	N	Neg.
10. 1943—Normal male infant	O	N	Neg.
11. 1944—Stillbirth (present pregnancy)			

The husband of this patient, in contrast to the fathers in Cases 1 and 2, is heterozygous for the Rh factor and belongs to genotype Rh<sub>2</sub>r<sub>h</sub>. It would therefore be expected that one-half of the children of this couple should be Rh positive (type Rh<sub>2</sub>), and one-half Rh negative. If the stillbirths are counted as Rh positive, a close approximation is obtained to the expected 1:1 ratio. In future pregnancies, this couple has a 50 per cent chance of having an Rh-negative and therefore normal child. Should such a fetus be Rh positive, it almost surely would be stillborn.

In this case it took more than one pregnancy with Rh-positive fetuses to sensitize the mother sufficiently to give rise to infants with hemolytic disease.

Nevertheless, the sensitization, when it finally did develop, was intense, as evidenced by the presence of blocking antibodies in the maternal serum and the occurrence of stillbirths in the seventh, eighth, and eleventh pregnancies. The mild jaundice exhibited by the female infant born after the fifth pregnancy was probably a manifestation of a very mild hemolytic disease. After the fifth pregnancy the sensitization became more pronounced so that only Rh-negative infants survived.

Of particular interest is the eighth pregnancy which yielded a pair of unlike-sexed twins. The male twin was Rh negative and normal, while the female twin died within thirteen hours from icterus gravis, undoubtedly because she was Rh positive.

CASE 4.—The patient was a male infant, 6 days of age, from the service of Dr. David A. Meyers at the Adelphi Hospital. The infant was the eighth born to his parents. The first seven children had been all normal at birth, and all were alive except the oldest, who had died as a result of an automobile accident. On the second day of life the patient was noticed to be jaundiced. The jaundice deepened and by the fifth day the hemoglobin concentration had dropped to 63 per cent, the red cell count was 2.9 millions, and the blood film showed 5 normoblasts per 100 white blood cells.

Blood tests on the parents and children showed the results given in Table XI.

TABLE XI

BLOOD OF	GROUP	MN TYPE	Rh TYPE	Hr REACTION
Father	O	M	Rh <sub>1</sub>	Neg.
Mother	O	M	Neg.	Pos.
1. Brother born 1931 (not alive)				
2. Sister born 1933	O	M	Rh <sub>1</sub>	Pos.
3. Sister born 1938	O	M	Rh <sub>1</sub>	Pos.
4. Sister born 1939	O	M	Rh <sub>1</sub>	Pos.
5. Sister born 1941	O	M	Rh <sub>1</sub>	Pos.
6. Brother born 1942	O	M	Rh <sub>1</sub>	Pos.
7. Sister born 1943	O	M	Rh <sub>1</sub>	Pos.
8. Patient	O	M	Rh <sub>1</sub>	Pos.

The maternal serum contained no detectable Rh agglutinins and gave only a questionable blocking reaction, but by the conglutination method, Rh antibodies of titer 16 were readily demonstrable, proving that the patient's mother was sensitized to the Rh factor and establishing the diagnosis of congenital hemolytic disease. By the conglutination technique Rh antibodies were also readily detected in the infant's plasma, and autoagglutination of the infant's red cells by his own plasma was demonstrable. This corroborates the remarks concerning the ease with which maternal univalent antibodies pass through the placental barrier into the fetal circulation.

A remarkable feature of this case is the large number of pregnancies required to induce Rh sensitization, despite the fact that every child was Rh positive. There is no doubt that the father is homozygous (genotype  $Rh_1Rh_1$ ) because he is Hr negative and every one of the seven children tested belongs to type Rh<sub>1</sub>. If this couple has any more children, such children are certain to have congenital hemolytic disease.

For treating the infant, 200 c.c. of blood were obtained from a Group O, Rh-negative professional donor. One-half of the blood was transfused at once and the remainder on the following day. The infant improved immediately, the jaundice cleared, and when the patient was seen a week later, the hemoglobin was 85 per cent. He was discharged from the hospital, and the parents were instructed to return with him in a week. He was not brought back until he was five weeks old, however, and by that time the hemoglobin had dropped to 40 per cent. Two hundred cubic centimeters of blood were then drawn from the patient's mother and the washed and packed red cells (about 80 c.c.) transfused by syringe and two-way stopcock into a scalp vein. The hemoglobin rise this time was sustained, and complete recovery followed.

The presence of Rh antibodies in the infant's serum, as demonstrated in this and other cases, is the rationale for the use of Rh-negative blood when treating infants with hemolytic disease, even though the infants themselves are Rh posi-

tive. When Rh-negative donors are not available, it is convenient to transfuse the mother's washed cells, and if the packed red cells are transfused, it is possible to get the effect of a transfusion of twice the volume of whole blood. The use of maternal washed cells is permissible even though the mother's blood belongs to an incompatible blood group, and preliminary grouping and Rh and cross-matching tests may be dispensed with if facilities for carrying out the tests are not available.

CASE 5.—A physician in Santiago, Chile, reported that his wife was in the sixth month of her third pregnancy and showed signs of hydramnios. He was concerned because he had found his wife to be Rh negative and himself Rh positive. He could not detect any Rh agglutinins in his wife's serum, but the patient gave the following significant obstetric history:

The first pregnancy occurred in 1943. About three months after marriage the patient had a ruptured ectopic gestation for which an operation was performed. After the operation she was given two transfusions of Group A bank blood, not selected for the Rh factor. There were no untoward reactions following these transfusions.

The second pregnancy occurred in 1944 and terminated with an eight-month macerated fetus.

Grouping and Rh tests were carried out on blood samples mailed from Chile, with the results given in Table XII.

No Rh agglutinins could be detected in the patient's serum, but blocking antibodies were present in high titer (50 units), and by the conglutination technique, Rh antibodies of titer 90 were demonstrable. In view of the high titer of Rh blocking antibodies in the patient's serum, and the fact that her husband was Hr negative, the prognosis for the present pregnancy was grave. In fact, the pregnancy soon did terminate with a seven-month stillbirth.

TABLE XII

BLOOD OF	GROUP AND SUBGROUP	MN TYPES	Rh TYPE	Hr REACTION
Husband	O	MN	Rh <sub>1</sub>	Neg.
Patient	A <sub>2</sub>	MN	Neg.	Pos.

There is no doubt that the two blood transfusions given to this patient were the cause of her sensitization to the Rh factor, because the ectopic pregnancy had not advanced far enough to bring about Rh sensitization. Had Rh-negative blood been transfused, most likely the second pregnancy would have yielded a viable infant. This case serves to illustrate the importance of Rh tests before giving blood transfusions to women in the child-bearing age, as already pointed out.

CASE 6.—The patient was an infant who became pale and then jaundiced shortly after birth, so that blood transfusion therapy was necessary. The infant was his mother's second child; the first pregnancy had terminated with a normal female child 4 years of age at this time.

Grouping tests on the infant, his parents, and sister gave the result listed in Table XIII.

These results came as a surprise, because we had felt reasonably sure from the clinical picture that the infant had congenital hemolytic disease. However, this diagnosis could now be excluded, especially since the maternal sera contained no abnormal isoantibodies.

TABLE XIII

BLOOD OF	GROUP	MN TYPE	Rh TYPE	Hr REACTION
Father	O	MN	Rh <sub>2</sub>	Pos.
Mother	O	M	Rh <sub>1</sub> Rh <sub>2</sub>	Pos.
Sister (4 years of age)	O	MN	Rh <sub>2</sub>	Pos.
Patient	O	M	Rh <sub>1</sub>	Pos.

The history was then obtained that the patient had had a traumatic delivery. He soon died, despite transfusion therapy, and an autopsy revealed the presence of a ruptured liver with hemorrhage into the parenchyma of the liver.

CASE 7.—This case was referred by Dr. A. M. Young, of the Mount Sinai Hospital in Cleveland, Ohio, because the patient, a newborn infant, had all the clinical signs and symptoms of congenital hemolytic disease, yet mother and infant were both Rh positive. In the maternal serum an irregular agglutinin was detected, which I was requested to identify.

Grouping and Rh tests showed the results given in Table XIV.

Thus, the infant's red blood cells contain all three Rh factors, Rh<sub>0</sub>, Rh', and Rh'', while the maternal red cells contain only factors Rh<sub>0</sub> and Rh'. The presence in the maternal serum of an atypical immune isoagglutinin of titer 32 was corroborated. As expected, this proved to correspond in specificity to anti-Rh''.

TABLE XIV

BLOOD OF	GROUP AND SUBGROUP	MN TYPE	Rh TYPE
Mother	A <sub>1</sub>	MN	Rh <sub>0</sub>
Infant	A <sub>1</sub>	MN	Rh <sub>0</sub> Rh <sub>1</sub>

While type Rh<sub>0</sub> mothers with type Rh<sub>0</sub> or type Rh<sub>0</sub>Rh<sub>1</sub> infants and type Rh<sub>1</sub> mothers with type Rh<sub>0</sub> or type Rh<sub>0</sub>Rh<sub>1</sub> infants are quite common, these combinations only rarely give rise to cases of congenital hemolytic disease, because factors Rh' and Rh'' are much weaker antigens than Rh<sub>0</sub>. As already mentioned, about 90 per cent of the clinical cases of congenital hemolytic disease can be traced to the Rh<sub>0</sub> factor.

CASE 8.—An infant 12 days old was seen in consultation with Dr. Benjamin Bloom, because of the sudden appearance of jaundice and pallor the day before. The patient was the first child. Her mother had never had a previous pregnancy and had never received a blood or plasma transfusion. The patient was born at term but her birth weight was only 4 pounds, 11 ounces. In all other respects she appeared normal and was discharged from the hospital on the eighth day. At home, she made satisfactory progress until jaundice and anemia appeared on the eleventh day. Blood count performed on the twelfth day showed: hemoglobin, 50 per cent; red blood cells, 1.75 million per cubic millimeter; white blood cells, 17,000; polymorphonuclears, 41 per cent; lymphocytes, 53 per cent; monocytes, 6 per cent; no nucleated red cells on smear.

Grouping and Rh test showed the results given in Table XV.

Since the patient's mother belonged to type Rh<sub>0</sub>Rh<sub>1</sub> and was Hr positive, this excluded the Rh factors and the Hr factor as having any bearing on the problem. The incompatibility in blood groups suggested that this might have some bearing on the problem, so the isoagglutinin titers of the maternal serum were determined. By the agglutination method the titers were about twenty-five times as high as the average normal titer, namely, the anti-A titer (for A<sub>2</sub> test cells) was 500, while the anti-B titer was 250. By the conglutination technique the titers were even higher, namely, 5,000 for anti-A and 500 for anti-B. In view of these findings, there could be no doubt but that this patient's hemolytic anemia was due to isoimmunization of the mother against the agglutigen A in the infant's blood.

TABLE XV

BLOOD OF	GROUP AND SUBGROUP	MN TYPE	Rh TYPE	Hr REACTION
Father	A <sub>1</sub>	N	Rh <sub>0</sub>	Pos.
Mother	O	MN	Rh <sub>0</sub> Rh <sub>1</sub>	Pos.
Patient	A <sub>1</sub>	N	Rh <sub>1</sub>	Pos.

Two of the questions raised by this case are how congenital hemolytic disease developed in a firstborn in the absence of previous sensitization of the mother by transfusion and why the onset of the disease was delayed until the eleventh day. Probably the high anti-A titer found in the tests did not accurately reflect the titer during pregnancy because, for reasons already pointed out, the isoantibody titer rises after delivery. Since the infant had been breast fed, she probably ingested larger and larger quantities of anti-A glutinins as the maternal isoantibody titer rose, and finally enough antibody was absorbed by the infant to bring about hemolysis. This serves to emphasize the

importance of interdicting breast feeding in all cases of congenital hemolytic disease. Another factor possibly delaying hemolysis was the infant's immaturity judging from its low weight, and the corresponding immaturity of its X protein. In another milder case of congenital hemolytic disease in a firstborn infant due to A-B isoimmunization, the mother gave the significant history that two years previously she had received two transfusions of reconstituted, desiccated pooled plasma. Experiments performed by me demonstrate that the intravenous injection of as little as 40 c.c. of such plasma can induce a pronounced rise in titer of the isoagglutinins anti-A and anti-B. This suggests that plasma transfusions should not be given indiscriminately to women in the child-bearing age, and it might also be mentioned that injections of therapeutic horse antisera may produce the same effect because such sera also contain A and B group substances.\*

Tests on the infant's saliva showed her to be a secretor, thus disproving the contention that only nonsecretors are subject to congenital hemolytic disease due to A-B isoimmunization.

The infant was given a transfusion of 60 c.c. of the packed, washed red cells of her mother. When the patient was seen three days later, her hemoglobin had risen to 66 per cent. By the end of the week, however, the hemoglobin had fallen to 45 per cent, so a second transfusion of washed maternal red cells was given. Again the patient improved, and this time recovery was sustained.

#### CONCLUSIONS

With the laboratory methods now available, it is possible to diagnose accurately practically every case of congenital hemolytic disease or to exclude this diagnosis. It is also possible to specify more exactly the pathogenesis, whether due to isoimmunization to one or more of the Rh factors, Rh<sub>0</sub>, Rh' and Rh'', or to the Hr factors or the A-B-O factors.

The great majority of erythroblastotic infants born alive can be saved by transfusions of Rh-negative blood or washed maternal red cells. However, no effective method is known for saving infants from the lethal effects of certain hepatic injuries sustained as a result of the disease or from kernicterus or hemorrhagic complications. What underlies the constitutional predisposition which makes one individual easier to sensitize to the Rh factor than another is not clearly understood.

Future work should be concerned primarily with the prophylaxis and treatment of congenital hemolytic disease. This problem will not be completely conquered until effective methods have been devised for treating sensitized Rh-negative women so that they are capable of bearing normal Rh-positive infants.

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\*For the same reason, the indiscriminate use of Group O blood neutralized with A and B group substances for transfusing women is not unobjectionable. Even diphtheria toxin-antitoxin or toxoid injections could stimulate a rise in the titers of the A and B isoantibodies.



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# LABORATORY METHODS

## STOMACH-TUBE FEEDING OF SMALL LABORATORY ANIMALS

A SIMPLE "HAIRPIN" GAG INSURING SAFE AND RAPID  
INTUBATIONS BY A SINGLE PERSON

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ACCURATE oral administration of measured amounts of a drug to laboratory animals is best achieved with the help of a stomach tube. As early as 1908 Marks<sup>1</sup> described the use of a silk rubber catheter for the stomach-tube feeding of mice. His procedure was soon widely adopted and at present is almost universally used. Most investigators substituted a simple metal cannula (made from an intramuscular injection needle) for the silk rubber tube. This eliminated the necessity of a mouth gag, since, unlike a rubber tube, the needle could not be punctured or severed by the animal's teeth during the process of insertion and withdrawal. Recently, Ferril<sup>2</sup> proposed a similar metal cannula, made from an injection needle by soldering a brass knob to its end, for the feeding of rats. However, while Mark's procedure works well in mice and even small rats, the less experienced operator will encounter difficulties in feeding full-grown and untrained rats without fixation of the jaws and without any other immobilization.

The procedure generally adopted today in most research laboratories makes use of a wooden mouth gag with a central hole for the gum elastic catheter.<sup>3,4</sup> The main drawback of this instrument is that it completely obscures the structures of the mouth and pharynx, so that the stomach tube has to be guided by touch rather than by sight. Thus difficulty may be encountered in attempts to pass the stomach tube above the animal's tongue. To overcome this obstacle, the tongue has to be forced and held forward between the gag and the animal's lower jaw. This requires that the gag be "put well back into the rat's mouth at the moment of insertion"<sup>3</sup> and usually also be rotated around its long axis counter clockwise when held with the right hand. This process is time consuming and, because of the crude shape of wooden mouth gags, is likely to hurt the animal and to stimulate violent struggling, which in turn may result in abrasion wounds of the gums. Obviously this is particularly undesirable if repeated intubations have to be carried out.

To obviate these difficulties, a simple instrument was devised which has proved its merits in more than six years of continuous use in many thousands of stomach-tube feedings of albino rats performed by my associates and me.<sup>5</sup> It consists of a piece of strong wire shaped in the form of a hairpin, the ends of which rest in a short metal bar. A metal rod, attached to the opposite side of this bar, permits fixation of the "hairpin" gag to a standard laboratory support. The dimensions and proportions of the instrument are evident from Fig. 1, which also illustrates stomach tubes of various sizes and makes. The

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gadget can be easily improvised for temporary use by forcing the branches of a strong hairpin into a cork stopper, which rests in a double jawed clamp. Without fixation and with appropriate increase in size, the instrument will serve with advantage for larger laboratory animals.

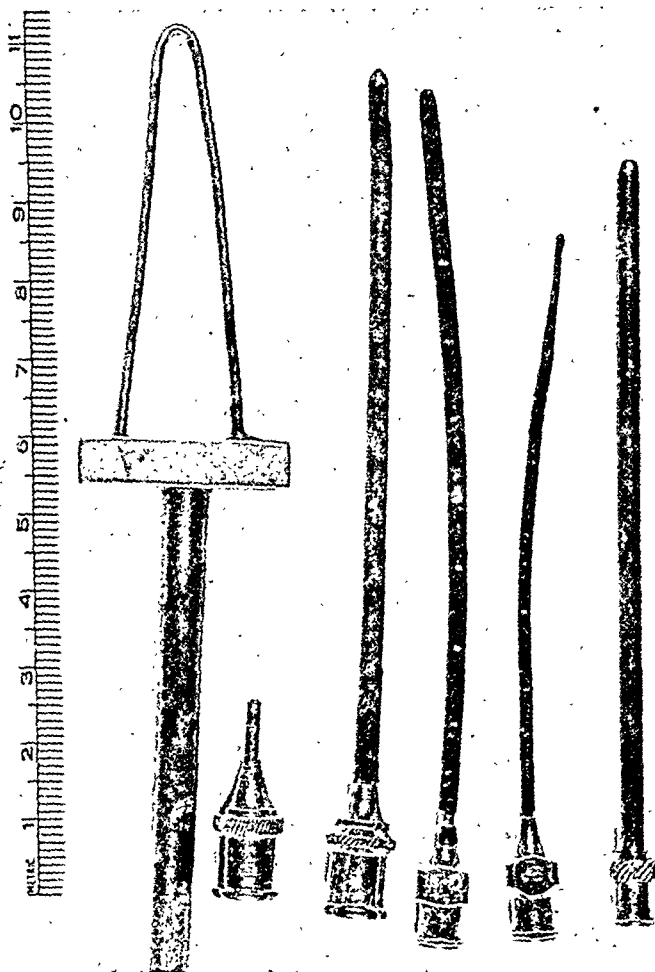


Fig. 1.—From left to right: "hairpin" gag, metal syringe adapter, soft rubber tube, silk rubber tube, silk rubber tube for small rats, metal cannula.

Before intubation, the rat is easily and quickly immobilized by wrapping it comfortably in a small towel (size of a large napkin, folded once) in such a way that the escape of the front limbs is prevented, whereas the head and neck remain free. The wrapping is best accomplished in the following manner: the folded towel, with the folding line pointing cranially, is placed over the animal's back not including the head; the rat is then grasped through the towel with the left hand and lifted into the air, while the right hand guides the left free end of the towel ventrally over the front legs, thus forming a "funnel" with its narrowest circumference around the animal's neck. Fixation of the towel at the neck is achieved with the help of a large artery clamp which is gently closed over the towel so as to include a part of the fur between its ends. This manipulation takes only a few seconds in actual performance.

The rat is now grasped firmly at the back of its head just behind the ears with the thumb and forefinger of the left hand, while the back of the middle finger presses against its vertebral column, thus enforcing extension of the

head and slight opening of the mouth. This grip completely immobilizes the animal. The towel, hanging like a cloak around its body, prevents scratching or violent movements. Under slight upward pressure maintained by the left hand throughout the intubation, the jaws of the rat can now be readily slid on sideways over the narrow end of the "hairpin."

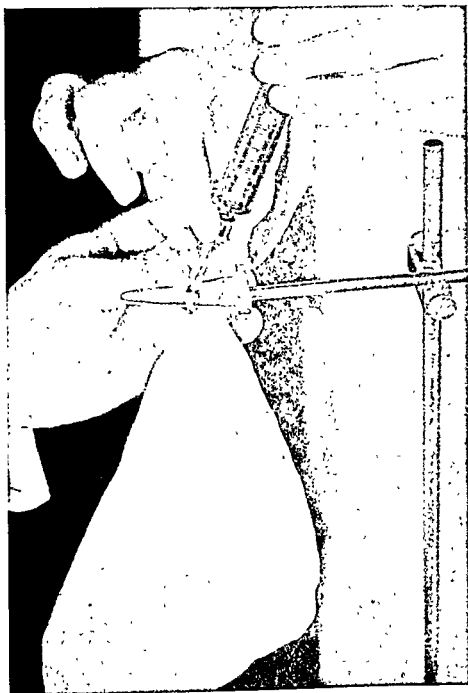


Fig. 2.—Stomach-tube feeding with assistance.

As is apparent from Fig. 2, fixation of the gag to the laboratory stand is carried out in such a way that the plane determined by the triangular device forms an angle of about 45 degrees with the horizontal. The wedgelike shape of the instrument allows good adjustment to the size of the animal's mouth. With the rat in feeding position, all structures of the mouth are exposed and clearly visible.

The syringe, containing the desired amount of drug, and the attached stomach tube are now grasped with the operator's right hand and without using force the tube is gently guided downward above the animal's tongue (Fig. 2) until its length is lost in the digestive tract and mouth; the plunger of the syringe is then forced down. The entire procedure can be carried out with remarkable speed and safety and with a minimum of discomfort to the animal.

The stomach tube is best made from the head part of a French ureteral silk woven or x-ray catheter which is cut off so as to contain the rounded tip

**Clinical Atlas of Blood Diseases.** By *A. Piney*, M.D., M.R.C.P., Physician, St. Mary's Hospital for Women and Children; and *Stanley Wyard*, M.D., F.R.C.P., Physician, The Royal Cancer Hospital, London, and Princess Beatrice Hospital, London, Sixth Edition. The Blakiston Company, Philadelphia, Pa. Price \$5.00. Cloth with 138 pages and 48 illustrations.

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### Announcement

Due to conditions beyond the control of the editors and publishers, several issues of the JOURNAL are printed on an inferior grade of paper. Just as soon as the standard good grade of paper is available, its use will be resumed.

# A COMPARISON OF THE RESULTS OBTAINED WITH THE HANGER CEPHALIN-CHOLESTEROL FLOCCULATION TEST AND THE MACLAGAN THYMOL TURBIDITY TEST IN PATIENTS WITH LIVER DISEASE

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**M**ACLAGAN has recently reported a study of the behavior of various human sera with a buffered thymol solution.<sup>1, 2</sup> This study was based upon the concept that globulins are precipitated more or less readily by phenolic compounds. MacLagan referred specifically to phenol as in the Pandy test and to cholesterol in the cephalin-cholesterol flocculation test of Hanger.<sup>3</sup> It was found that other phenolic compounds did, in fact, precipitate globulin. Thymol was the most satisfactory of any of a number tried. Employing a saturated aqueous solution of thymol buffered with barbitone and sodium barbitone to a pH of 7.8, MacLagan found that essentially the same results were obtained with various normal and pathologic sera as with the colloidal gold test, in which the detection of increased amounts of globulin depends upon precipitation of the gold. The turbidity occurring in abnormal sera when brought together with the buffered thymol solution was shown to be due to precipitation of a complex containing protein, thymol, cholesterol, and a phospholipid of lecithin type. MacLagan measured the degree of turbidity by comparing the solution after one-half hour with the formazin standards devised by Kingsbury and associates<sup>4</sup> and used for many years in the rough quantitative determination of protein in the urine. Using this method, MacLagan found that normal sera ranged from 0 to 4 units.

Number of units =  $\frac{\text{Standard tube reading} \times \text{final dilution of serum}}{600}$ , the standard dilution being 1 to 60 (0.05 c.c. of serum and 3.0 c.c. of the buffered thymol solution)

In 120 of 130 cases of hepatitis (stage of disease not given), significantly positive values above 4 units were observed. The same was true in all of thirteen cases of cirrhosis of the liver. However, in thirty-seven cases of jaundice due to extrahepatic biliary obstruction, only three exhibited values above 4 units; these were in the 5 to 7 range.

Assuming that MacLagan's thymol turbidity test was to provide essentially the same information as the cephalin-cholesterol flocculation test of Hanger, it would offer a number of important advantages, including (1) an easily prepared stable solution containing weighed amounts of pure chemicals, (2) completion in one-half hour instead of forty-eight hours, and (3) a simple method

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of estimating the degree of positivity. Although Maclagan implied that the basis of the thymol turbidity test is identical with that of the cephalin-cholesterol flocculation, he did not present any data to reveal whether the results with the two methods are equivalent.

The purpose of the present communication is to present data obtained by comparing the cephalin-cholesterol flocculation test and the thymol turbidity test as applied to various human sera. The cephalin-cholesterol flocculation test was performed according to Hanger's method, employing Difco\* antigen. In carrying out the thymol turbidity test, 0.1 c.c. of serum was diluted to 6 c.c. with the thymol buffer solution, rather than 0.05 to 3, as according to Maclagan; in other respects the procedure was identical. The turbidity of the final solution is estimated by comparison with Kingsbury's standards.<sup>4</sup> These consist of ten tubes with degrees of turbidity corresponding to a range of from 10 to 100 mg. Prepared tubes obtained on the market† have been found to be satisfactory except that the only concentrations included above 50 mg. per cent are the 75 and 100 mg. per cent tubes. This requires that sera producing turbidity greater than that of the 50 mg. per cent tube must be diluted further.

The data which will be considered are based upon 252 Hanger and Maclagan tests carried out on samples of blood serum from 145 individuals and may be divided as follows:

GROUP	NUMBER OF INDIVIDUALS	NUMBER OF TESTS
I—Presumably normal adults	31	31
II—Hepatitis	60	154
III—Cirrhosis and subacute atrophy	13	19
IV—Extrahepatic biliary obstruction		
Calculous	5	5
Cancer	6	8
Stricture	1	1
V—Hepatic carcinomatosis	4	4
VI—Inoculation malaria	6	10
VII—Miscellaneous	18	20

In this study the diagnosis was based on clinical features alone in the patients in Groups I, II, and VI, while in those included in Groups III, IV, and V the diagnosis was established either at operation or autopsy, or by means of liver biopsy.

#### RESULTS

The data are shown in Tables I to VII, inclusive. It is seen that, in the main, there is a distinct correlation between the two tests. If one accepts Maclagan's value of 4 units for the upper limit of a negative test and, similarly, for the Hanger test a value of a trace for twenty-four hours or 1 plus for forty-eight hours, twenty-seven instances are found in the present data in which one test was positive and the other negative. In other words, there was agreement in 225 of 252 tests, or 89.3 per cent, on the arbitrary basis just mentioned. The twenty-seven instances of disagreement are brought together in Table VIII. It is seen that the majority of these are borderline. In the patients with hepatitis the discrepancies noted were of little moment as they were associated with transitions in the course of the disease either toward recovery or relapse, usually the former (see Table II). In each instance the trend of each test and the information gained were essentially the same.

\*Difco Laboratories, Inc., Detroit, Mich.

†Standard Reagents Co., Philadelphia, Pa.



More serious discrepancies are encountered in Groups III, IV, and V, as noted in Table VIII. In both cases of subacute atrophy distinctly positive Hanger tests with negative thymol turbidity were exhibited. A discrepancy was noted in but one of the eleven cases of cirrhosis, and it is perhaps of some interest that this was the only example of "alcoholic" cirrhosis in the group. The other cases were of unknown etiology or represented transitions from infectious hepatitis, cases which have been referred to as "cholangiolitic cirrhosis."<sup>5</sup> As noted in Table III there was also one example of hemochromatosis and one which was first thought to represent the so-called xanthomatous biliary cirrhosis; however, at autopsy the latter was shown to be only an ordinary portal cirrhosis.<sup>6</sup>

But ten cases of proved cancer are represented in the series and it is interesting that there were varying discrepancies in six of these, as given in Table VIII. A rather remarkable reversal in Case 6 (Group IV) occurred within a period of three weeks. The significance of this is not at all clear.

Among the 89 per cent in whom both tests were positive, according to the arbitrary division referred to in the foregoing, many instances of poor quantitative correlation were encountered; for example, sera exhibiting a 3 plus, 4 plus Hanger test with but 5 or 6 units of thymol turbidity. Conversely there are a number of examples of a 2 plus, 3 plus Hanger test with 10 units or more of thymol turbidity. Case 14 in Group II, hepatitis (Table II), illustrates the type of variation in relative discrepancy that may be encountered especially when serial tests are being performed. The first result in this case was a Hanger test of 1 plus, 2 plus, and a MacLagan test of 10 units; sixteen days later the Hanger test was 2 plus, 3 plus and the MacLagan test was 8 units. In Cases 55 and 56, in the same group, it is seen that at certain periods Hanger tests of 3 plus, 4 plus and MacLagan tests of 5 units were observed. Other and similar lack of quantitative correlation is frequently observed in the tables.

TABLE I. GROUP I—PRESUMABLY NORMAL ADULTS

CASE	HANGER TEST		MACLAGAN TEST IN UNITS
	24 HR.	48 HR.	
1	0	0	2
2	0	0	2
3	0	0	2
4	0	0	3
5	0	0	3
6	0	0	3
7	0	0	2
8	0	0	4
9	0	0	3
10	0	0	3
11	0	0	3
12	0	0	1
13	0	0	1
14	0	0	1
15	0	0	1
16	0	0	1
17	0	0	1
18	0	0	1
19	0	Trace	1
20	0	0	1
21	0	0	1
22	0	0	1
23	0	0	1
24	0	0	1
25	0	0	1
26	0	0	1
27	0	0	1
28	0	0	1
29	0	0	1
30	0	0	1
31	0	0	1

TABLE II. GROUP II—HEPATITIS

CASE	DATE	HANGER		MAC- LAGAN TEST IN UNITS	CASE	DATE	HANGER		MAC- LAGAN TEST IN UNITS
		24 HR.	48 HR.				24 HR.	48 HR.	
1	2/16	0	1+	4	16	2/16	3+	4+	10
	2/17	1+	1+	5		2/19	3+	4+	12
	2/25	1+	2+	6		2/21	2+	3+	10
	3/ 3	1+	2+	6		2/26	2+	3+	7
	3/ 5	2+	3+	8	17	2/16	3+	4+	8
	3/ 7	2+	3+	9		2/21	2+	3+	9
2	3/19	2+	2+	7		3/3	3+	3+	9
	2/20	0	0	1	18	3/19	1+	1+	6
	2/27	0	0	4		3/1	2+	3+	8
3	3/3	0	0	2		3/10	3+	4+	10
	2/19	0	0	3	19	4/3	2+	2+	7
	2/21	0	0	4		4/9	1+	1+	5
4	3/17	0	0	2	20	2/26	0		4
	2/16	0	1+	5		2/28	0		2
	3/13	0	0	2	21	3/3	0	0	3
5	3/17	0	0	3		2/22	1+	1+	5
	3/23	0	0	2	22	2/27	1+	1+	5
	3/16	0	0	4		3/3	1+	1+	4
	3/20	1+	1+	6	23	3/20	0	0	3
6	3/29	0	1+	5		3/21	0	0	2
	4/2	0	0	4	24	2/20	0		3
	2/16	1+	1+	6		2/21	0		2
	2/19	1+	2+	6	25	2/15	0	0	3
7	3/10	0	1+	5		2/17	0	0	2
	3/15	0	0	4	26	2/26	2+	3+	8
	3/29	0	0	3		3/6	1+	1+	7
8	3/20	0	0	3		3/12	0	0	4
	2/22	0		4	27	3/17	0	0	3
9	3/31	0	0	3		2/20	3+		8
	3/16	1+	2+	7		2/27	1+	2+	7
	3/29	1+	2+	6	28	3/6	1+	2+	6
	4/4	1+	1+	5		3/10	0	0	3
10	4/9	0	1+	5		2/26	1+	1+	3
	2/22	0		4	29	3/13	0	0	3
	3/5	0		2		3/15	0	0	2
11	3/29	0	0	3	30	3/6	0		3
	2/22	0		3		3/9	0	0	3
12	3/3	0		2	31	4/9	0	0	4
	2/15	1+	1+	5		4/12	0	0	3
	2/26	1+	1+	4	32	3/6	1+	2+	5
	3/5	0	0	3		3/15	1+	2+	7
	3/14	0	1+	4		3/17	2+	3+	8
	3/16	1+	1+	5	33	3/8	0	0	2
13	3/19	0	1+	5		2/26	0		4
	2/5	1+	2+	10	34	3/3	0		2
	2/10	2+	3+	10		3/5	0		3
	2/21	2+	3+	8	35	3/1	0		3
	2/23	1+	2+	8		2/22	1+	1+	6
	2/27	1+	2+	7	36	3/1	0	0	4
	3/3	2+	2+	7		3/3	0	1+	5
	3/9	2+	3+	7		3/5	1+	1+	5
14	3/16	2+	3+	8	37	3/19	0	0	3
	3/8	2+	3+	9		2/22	0		2
	3/10	2+	3+	10	38	2/22	0		2
	3/14	2+	2+	7		2/22	0		2
	3/19	0	0	4		2/22	0		2
	3/28	0	0	3		2/22	0		2

CASE	DATE	HANGER		MAC- LAGAN TEST IN UNITS	CASE	DATE	HANGER		MAC- LAGAN TEST IN UNITS
		24 HR.	48 HR.				24 HR.	48 HR.	
39	2/19	0		3	49	3/8	3+	4+	9
40	2/28	0		3		3/19	2+	3+	5
41	2/22	0		3		3/21	3+	4+	7
						4/23	0	0	2
42	6/13	3+	4+	10	50	6/2	1+	2+	4
	6/20	2+	3+	7	51	6/20	3+	4+	8
	7/6	2+	3+	12	52	6/11	2+	2+	7
43	6/20	2+	3+	6		6/18	1+	2+	5
	6/27	2+	3+	7	53	3/16	3+	4+	7
44	7/5	3+	3+	16	54	3/7	1+	2+	6
	7/11	3+	4+	18	55	3/8	3+	4+	5
45	6/27	2+	3+	16		3/14	2+	3+	2
		2+	2+	20		3/16	3+	4+	3
		1+	2+	16		4/23	0	1	2
		1+	2+	15	56	4/10	3+	4+	5
46	6/2	0	Trace	1		4/12	2+	3+	5
47	3/12	3+	4+	6			0	Trace	2
	3/16	3+	4+	10	57	4/21	2+	3+	8
		2+	2+	7		4/25	2+	2+	5
		1+	2+	5	58	4/10	2+	3+	5
		0	1+	4	59	5/16	1+	1+	2
48	1/25	2+	3+	8	60*	4/10	0	Trace	4
		3+	4+	9					

\*Postarsphenamine.

TABLE III. GROUP III—CIRRHOSIS AND SUBACUTE ATROPHY

CASE	DATE	HANGER TEST		MACLAGAN TEST IN UNITS
		24 HR.	48 HR.	
1*		3+	4+	10
2†	5/16	Trace	1+	1
	5/31	0	1+	1
3	5/29	3+	4+	12
4	6/11	0	1+	3
5	5/18	3+	4+	12
	5/23	3+	4+	12
	5/25	3+	4+	12
	5/28	3+	4+	14
	7/11	2+	3+	24
	8/24			30
6	6/12	1+	2+	3
7	1/29	0	1+	5
8	2/9	3+	4+	7
9	7/25	1+	1+	8
10	8/17	2+	3+	2
11	8/20	4+	4+	16
12†	6/9	2+	3+	1
13†	4/19	3+	3+	2

\*With hypercholesterolemia and xanthomata; autopsy revealed Laennec cirrhosis without evidence of so-called xanthomatous biliary cirrhosis.\*

†Hemochromatosis.

‡Subacute atrophy.

TABLE IV. GROUP IV—EXTRAHEPATIC BILIARY OBSTRUCTION

	CASE	DATE	HANGER TEST		MACLAGAN TEST IN UNITS
			24 HR.	48 HR.	
Cancer	1	1/8	0	0	3
	2	5/16	1+	2+	1
	3	6/6	0	0	1
	4	1/23	Trace	1+	2
	5	1/25	2+	3+	7
	6	6/22	0	Trace	2
Calculous		7/2	0	1+	5
		7/24	3+	4+	1
	7	5/2	Trace	Trace	1
	8	3/17	0	0	3
	9	7/12	Trace	1+	1
	10	7/2	0	Trace	1
Stricture	11	7/18	0	0	1
	12	5/30	0	0	1

TABLE V. GROUP V—HEPATIC CARCINOMATOSIS

CASE	DATE	HANGER TEST		MACLAGAN TEST IN UNITS
		24 HR.	48 HR.	
1	1/30	1+	2+	3
2	3/16	2+	3+	2
3	3/10	1+	2+	3
4	8/6	1+	2+	2

TABLE VI. GROUP VI—INOCULATION MALARIA

CASE	CHILLS	DATE	HANGER TEST		MACLAGAN TEST IN UNITS
			24 HR.	48 HR.	
1	Before	1/27	0	0	2
	During	2/20	3+	4+	5
2	Before	1/2	0	0	2
	During	1/19	2+	3+	7
3	During	1/11	3+	4+	8
	During	1/24	3+	4+	14
4	During	12/23	3+	4+	7
	After	1/23	1+	2+	2
5	After	1/23	0	0	2
6	After	1/30	0	0	2

## DISCUSSION

The present results indicate quite clearly that the underlying basis of the Hanger and MacLagan tests is not identical. Although an agreement was noted in 89 per cent of the tests, it is recognized that discrepancies in degree of positivity were frequent. Such discrepancies preclude any general substitution of the MacLagan for the Hanger test, at least in so far as routine use for differential diagnosis is concerned. The present study indicates that the MacLagan test is probably more often negative than the Hanger test in patients with serious diffuse liver disease, such as subacute yellow atrophy and certain forms of cirrhosis. Further study may reveal that certain forms of cirrhosis are more likely to exhibit negative thymol turbidity and positive cephalin flocculation. It has already been noted that the only case in the present series in which this was observed was one of alcoholic (fatty) cirrhosis. The present data suggest that the thymol turbidity test is more often negative and hence less likely to offer confusion in diagnosis in cases of hepatic or biliary tract cancer. In so far as the hepatitis problem is concerned, the present data reveal

TABLE VII. GROUP VII—MISCELLANEOUS

CASE	CONDITION	DATE	HANGER TEST		MACLAGAN TEST IN UNITS
			24 HR.	48 HR.	
1	Infectious mononucleosis	3/16	1+	2+	1
		3/20	2+	3+	6
2	Hepatic vein thrombosis (polycythemia)	1/23	1+	2+	2
3	Pneumonia and empyema	1/22	Trace	1+	2
4	Cholelithiasis	3/7	0	0	2
5	Tuberculous peritonitis?	3/7	Trace	1+	3
6	Pneumococcus pneumonia with jaundice and numerous spider nevi	4/20	0	0	4
		5/2	0	0	2
7	Hemolytic jaundice	6/15	2+	3+	2
8	Rheumatoid arthritis	5/26	0	0	3
9	Hypochromic anemia	6/18	1+	2+	3
10	Inferior vena cava thrombosis (metastatic cancer?)	6/18	2+	3+	10
11	Hypercholesterolemia	6/18	0	0	1
12	Hemolytic jaundice	1/30	1+	1+	3
13	Pancreatic adenoma	7/24	0	0	1
14	T-tube drainage of common duct	8/6	0	0	2
15	Cholelithiasis	8/22	0	Trace	3
16	C.N.S. syphilis	1/23	Trace	1+	2
17	C.N.S. syphilis	3/9	0	1+	2
18	C.N.S. syphilis	1/16	0	Trace	1

TABLE VIII. DISAGREEMENT BETWEEN TESTS

	CASE	HANGER TEST		MACLAGAN	
		24 HR.	48 HR.	TEST IN UNITS	
Group II—Hepatitis	4	0	1+	5	
	5	0	1+	5	
	6	0	1+	5	
	9	0	1+	5	
	13	0	1+	5	
	21	1+	1+	4	
	28	1+	1+	3	
	37	0	1+	5	
	50	1+	2+	4	
59	1+	1+	2		
Group III—Cirrhosis	6	1+	2+	3	
	7	0	1+	5	
	10	2+	3+	2	
	12	2+	3+	1	
	13	3+	3+	2	
Group IV—Biliary obstruction (cancer)	2	1+	2+	1	
	6	0	1+	5	
		3+	4+	1	
Group V—Hepatic carcinomatosis	1	1+	2+	3	
	2	2+	3+	2	
	3	1+	2+	3	
	4	1+	2+	2	
Group VI—Inoculation malaria (after fever)	4	1+	2+	2	
Group VII—Miscellaneous					
	Infectious mononucleosis	1	1+	2+	1
	Hepatic vein thrombosis	2	1+	2+	2
	Hemolytic jaundice	7	2+	3+	2
	Hypochromic anemia	9	1+	2+	2
	Hemolytic jaundice	12	1+	1+	3

that the Maclagan test is fully as useful and reliable as the Hanger test once the disease is well established and during its defervescence. We have not had opportunity to compare the tests during the preicteric or prodromal stage. It is quite possible that the Hanger test may become positive earlier than the Maclagan, especially if the present results in the two cases of subacute atrophy can be correlated with the evidence of early diffuse hepatocellular injury in infectious hepatitis.<sup>7</sup> This question should be studied at the earliest possible moment since the Maclagan test, if equally reliable in the early stage of the disease, is much easier to use on a large scale; for example, during an epidemic when extensive screening for early cases is much to be desired.

Because of its relative simplicity and rapidity, the Maclagan test can certainly be employed to good advantage as a routine procedure; the Hanger test may be reserved for cases in which the diagnosis is still in doubt, or where the result of the Maclagan test disagrees with other procedures such as the one-minute and total serum bilirubin,<sup>8</sup> the urine Ehrlich test for urobilinogen,<sup>9</sup> and the 5 mg. per kilogram forty-five minute bromsulfalein test.<sup>10</sup> The Maclagan test is also very helpful for serial usage where it has once been shown to be positive, as in cases of hepatitis. In other words, it has considerable value in following the course of the disease in any particular instance.

As noted at the outset, Maclagan believed that the thymol turbidity is due to a globulin-thymol-lipid complex and that the test gives essentially the same results as the colloidal gold procedure which has been used in the study of liver disease. The earlier studies of Hanger and his associates likewise implicated a globulin, that is, the gamma globulin, as the important factor in the cephalin-cholesterol flocculation.<sup>11</sup> Very recently, however, Hanger and co-workers<sup>12</sup> described evidence indicating that an alteration in character of the serum albumin must be present, together with an increased globulin, before flocculation will take place. The addition of normal albumin to sera high in globulin content was found to prevent flocculation. This may well constitute an essential difference between the mechanisms of the Hanger and Maclagan tests, a difference which would readily explain the discrepancies noted in the present study. Such a difference might also explain the positive Hanger and negative Maclagan tests exhibited by normal sera of dogs and other species.\*

#### SUMMARY AND CONCLUSIONS

1. The Hanger cephalin-cholesterol flocculation test and the Maclagan thymol turbidity test have been compared on 252 blood sera from 145 individuals. Thirty-one of these subjects were believed to be normal, while sixty (154 tests) were suffering from infectious hepatitis. In accordance with the previous findings of Hanger and Maclagan, the values in the normals did not exceed a trace (24 hr.) or 1 plus (48 hr.) for the cephalin flocculation or 4 units for the thymol turbidity.

2. Employing this as an arbitrary dividing line between positive and negative, an agreement of 89.3 per cent was noted. The majority of the 10.7 per cent of discrepancies between positive and negative were borderline and, in the hepatitis group especially, were not serious. In the cases of cirrhosis, subacute atrophy, and cancer, however, several serious discrepancies were encountered. In addition, a number of marked discrepancies in degree of positivity within the 89 per cent of correlation was observed.

3. It is evident that the underlying mechanism of the two tests is not identical and that the Maclagan test cannot be used interchangeably with, or

\*Personal communications from Dr. F. M. Hanger and Dr. S. Schwartz.

as a complete substitute for, the Hanger test. Because of its simplicity, stability of reagents, and rapidity, it can be used to advantage for serial observations where the diagnosis has been established, as, for example, in cases of infectious hepatitis. Information is needed as to the relative behavior of the tests in the preicteric or prodromal stage of this disease.

4. The MacLagan test can also be used to advantage in the differential diagnosis of jaundice or liver disease, as long as its limitations are recognized. The present study indicates that a strongly positive test is just as indicative of liver injury or disease as is the Hanger test and that it may prove to be more uniformly negative in cases of jaundice due to biliary tract cancer and in cases of hepatic cancer with or without jaundice. Conversely, it appears that the Hanger test is more often positive in cases of cirrhosis or subacute atrophy. Additional data are needed to determine this point and to reveal whether the thymol turbidity test is more likely to be negative in one type of cirrhosis than another.

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# ANTIGENIC PROPERTIES OF VARIOUS LIPOIDS FROM BEEF HEART

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## INTRODUCTION

**A**LL efforts to make the serodiagnosis of syphilis absolutely infallible and perfectly specific have failed up to the present time. Nearly forty years of research have made it clear that there is still a discrepancy between the different methods with regard to sensitivity and specificity. The Washington Serology Conference (1941) tried to evaluate the older and also some of the more recently published American methods. The findings were not uniform; Mahoney<sup>1</sup> attributed the discrepant results to "the different sensitivity of the tests; to the weakness in the test mechanics when the concentration of the reacting substance is in the region of the threshold of the test; to the presence of certain factors which have the power of determining the test or tests which may yield a positive reaction while others are negative." Not one of the thirty-one separate procedures was absolutely specific for syphilis.

Only Hinton flocculation and the Mazzini complement fixation procedures gave entirely negative results with sera from patients with malaria. But not one of the thirty tests was reliable when used with sera from patients with leprosy. Relatively, the best were the Mazzini complement fixation test (specificity 86.2 per cent) and the Boerner-Jones-Lukens simplified complement fixation test (83.7 per cent).

To the many recognized diseases of nonsyphilitic origin which sometimes give a positive syphilitic reaction, the following have recently been added: hyperproteinemia (Cardon and Atlas<sup>2</sup>) and immunization against various diseases (Arthur and Hale,<sup>3</sup> Kahn and associates,<sup>4</sup> Lubitz,<sup>5</sup> and Rein and Elsberg<sup>6</sup>). The question of the so-called false positive reaction is still under discussion and unsolved. Actually a false positive reaction does not exist. There is a positive reaction in patients without syphilis (nonspecific) and there is a positive reaction due to a technical error in a nonsyphilitic person. The latter will become negative when repeated; the former will stay positive. There is, therefore, abundant reason for making further attempts to reduce the number of nonsyphilitic positive reactions. From the various ways of approaching this problem, one seems to be an improvement of the antigens.

It is generally accepted that human serum contains more than one reagin. In an editorial, four types of reagins are described.

1. The flocculating factor present in minute traces in many human sera.
2. The "reagin" present in the occasional human serum in amounts sufficient to give a positive or doubtful test.
3. The "reagin" elaborated in the course of such nonsyphilitic diseases as leprosy, malaria, cowpox, and infectious mononucleosis.
4. The "reagin" elaborated in the course of syphilitic infection. It can be added that the latter is composed of two kinds: (1) "reagins" against the

From the Mount Sinai Hospital.

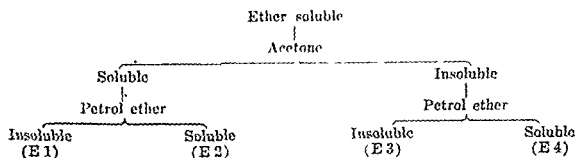
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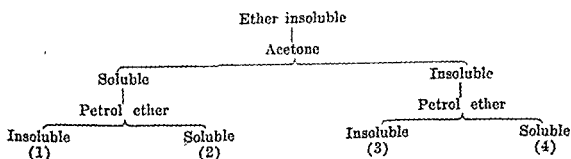
spirochetal lipoids as proved through the specific value of Gachtgens<sup>18</sup> and Klopstock's<sup>9</sup> spirochetal antigen and (2) the much more numerous nonspecific "reagins" which are detected with the commonly used beef heart antigens. Antigens are of very complex nature. To divide them into various parts and test their special action upon syphilitic and nonsyphilitic sera is the object of the following experiments.

#### TECHNICAL PROCEDURE

*Experiment 1.*—Twenty grams of dried beef heart powder\* were extracted twice with 200 c.c. of anesthesia ether at 8° C. After evaporation of the ether, the residual lipoids were divided into acetone-soluble and acetone-insoluble fractions by dissolving them in 500 c.c. of acetone at 50° C. Each of these was extracted with petrol ether at room temperature, yielding an acetone-soluble-petrol ether-insoluble fraction (E 1), an acetone-soluble-petrol ether-soluble fraction (E 2), an acetone-insoluble-petrol ether-insoluble fraction (E 3), and an acetone-insoluble-petrol ether-soluble fraction (E 4).

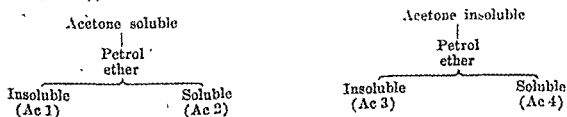


Remaining beef heart powder after extraction with ether was then repeatedly and completely extracted with acetone at 50° C., and the acetone-soluble fraction was divided into a petrol ether-soluble fraction (2) and a petrol ether-insoluble fraction (1) by dissolving it in 200 c.c. petrol ether three times. The acetone-insoluble fraction was extracted three times with 200 c.c. petrol ether at room temperature (4) and the remaining acetone- and petrol ether-insoluble fraction was dissolved in absolute alcohol at 50° C. (3).



The final antigen was prepared in the proportion 1:200 with absolute alcohol with the addition of 2 c.c. of 1 per cent cholesterol in alcohol to each 100 c.c. absolute alcohol.

*Experiment 2.*—Without a previous ether extraction, 10 Gm. of dried beef heart powder\* were extracted first with acetone and then with petrol ether to obtain an acetone-soluble-petrol ether-insoluble fraction (Ac 1), an acetone-soluble-petrol ether-soluble fraction (Ac 2), an acetone-insoluble-petrol ether-insoluble (Ac 3), and an acetone-insoluble-petrol ether-soluble fraction (Ac 4).



Another 10 Gm. of dried beef heart powder\* were first extracted with petrol ether and then with acetone to get an acetone-soluble-petrol ether-insoluble fraction (Pe 1), an acetone-soluble-petrol ether-soluble fraction (Pe 2), an acetone-insoluble-petrol ether-insoluble fraction (Pe 3), and an acetone-insoluble-petrol ether-soluble fraction (Pe 4).



As with the antigens in Experiment 1, these were dissolved in absolute alcohol in the proportion 1:200 with the addition of 2 c.c. of 1 per cent cholesterol in alcohol to each 100 c.c. absolute alcohol.

Antigens for Experiments 1 and 2 were stored for three months at room temperature before being tested. The tests were performed using the active method (Hecht<sup>10</sup>) complement fixation technique.

The proper titrated amount of the antigen is measured into a wide-mouthed bottle. A 10 c.c. pipette is introduced into the bottom of the bottle and the saline solution allowed to flow out very slowly so that the antigen will be raised. Then the bottle is placed in the incubator (37° C.) without shaking so that the overlying alcoholic antigen can evaporate.

The next day, after the alcohol has evaporated, the bottle is well shaken, and the evaporated part is replaced by distilled water and the required amount of saline added to obtain the proper dilution. For example, if the dose of the antigen is 0.2 c.c. of 1:25 dilution in saline, 1.0 c.c. of antigen is taken and 10 c.c. of saline is placed under it; the next day distilled water is added to make 10 c.c.; saline is then added to make a total of 25 c.c.

The titration of the antigen must be made also with an alcohol-free emulsion prepared as described.

The use of an alcohol-free antigen has two objects and advantages; first, to eliminate the damaging influence of the alcohol upon the complement and, second, to avoid the nonspecific precipitation of an alcoholic antigen when mixed with sera from patients with pneumonia, tuberculosis, and other febrile conditions.

To determine the sensitivity of the antigens, pooled sera were used as shown in Table I.

TABLE I. POOLED POSITIVE AND NEGATIVE SERA MIXED IN VARIOUS AMOUNTS TO OBTAIN A MEDIUM POSITIVE SERUM

POS. (C.C.)	SERUM (C.C.) NEG.	KLINE TEST		
		DIAG.	CONTROL	EXCLUSION
0.1	1.9	—	++	+++
0.2	1.8	±	++, +++	++++
0.3	1.7	+, ++	+++	++++
0.4	1.6	+++	++++	++++
0.5	1.5	++++	++++	++++

For the next test, the sensitivity test, a mixture of 0.3 c.c. syphilitic and 1.7 c.c. negative serum were taken (Table II).

After establishing the appropriate dilutions of the sixteen tested antigens, the sensitivity test was undertaken with at least 30 sera from patients with various forms of syphilis and with other diseases as well as from normal

\*Difco Laboratories, Inc., Detroit, Mich.

TABLE II. SENSITIVITY TEST  
(TESTED SERUM.—KLINE TEST: DIAGNOSTIC +, ++; CONTROL, +++; EXCLUSION, ++++)

DILU- TION OF ANTIGEN	E1	E2	E3	E4	1	2	3	4	AO1	AO2	AO3	AC4	PE1	PE2	PE3	PE4
1:100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:50	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-
1:80	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-
1:70	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-
1:60	+	+	-	-	-	-	-	-	+	+	+	+	-	+++	-	-
1:50	+	+	-	-	-	-	-	-	+	+	+	+	-	+++	-	-
1:40	+	+	-	-	-	-	-	-	+	+	+	+	+	+++	-	-
1:30	+++	+++	-	+	-	+	-	-	+++	+++	++	+++	+	+++	+	+
1:20	+++	+++	-	++	-	++	+	-	+++	+++	+++	+++	++	+++	++	++
1:10	++++	++++	+	++	+	++++	++	+	++++	++++	++++	++++	++++	++++	++++	++++
1:5	++++	++++	+	+++	++	++++	++	+	++++	++++	++++	++++	++++	++++	++++	++++

TABLE III. SPECIFICITY TEST

DIAGNOSIS AND KLINE TEST*	E1	E2	E3	E4	1	2	3	4	AO1	AO2	AO3	AC4	PE1	PE2	PE3	PE4
Treated syphilis +, ++, +++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Active syphilis ++++, +++++	+	+++	++	+++	+	++	+	+	++++	++++	+++	++++	++	++++	++	++
Latent syphilis +, ++, +++, +++++	+	++	+	-	+	++	+	+	+++	++	-	-	+	++	-	-
Cord blood	-	+	-	++	++	+	+	-	+	-	-	-	-	-	-	-
Uterine blood	++	+	+	++	++	+	-	-	-	-	-	-	+	-	-	-
Latent syphilis -+, -++	+	+	+	+	+	+	-	++	++	++++	+	+++	++	++	+	+
Carcinoma of uterus	-	+++	-	-	-	-	-	-	+	-	++	+	-	-	+	-
Pneumonia	-	++	-	-	-	-	-	-	+	-	++	+	-	-	+	-

\*Kline results are recorded here in three columns; they refer to diagnostic, control and exclusion tests, respectively.

TABLE IV. CHEMICAL COMPOSITION AND CLINICAL VALUES OF SIXTEEN ANTIGENS STUDIED

20 GM. BEEF HEART POWDER																	
REMAINING ETHER EXTRACTED POWDER																	
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patients. Examples are given in Table III. The antigen emulsions were used in the dose of 0.2 c.c. of the evaluated dilution.

According to their efficiency as syphilitic antigens, the sixteen antigens may be divided into four groups:

1. The best antigens were Pe 2 and Ac 2, each acetone-soluble-petrol ether-soluble fractions prepared without previous ether extraction. Ac 4, which is acetone soluble but petrol ether insoluble, was nearly as efficient as Ac 2.

2. Of medium value were Ac 1, E 2, E 1, and Ac 3, the three first mentioned being acetone soluble. They all had a tendency to react positively with sera from patients with nonsyphilitic diseases.

3. The equally efficient antigens 2, Pe 1, and Pe 4 were not sensitive enough and were nonspecific.

4. The remaining six antigens were very weak and nonspecific. (See Table IV.)

The most important part in every serodiagnostic test for syphilis is the specificity. The more sensitive the tests became, the more they approached the dangerous zone of nonspecific reactions. As shown in a previous paper,<sup>10c</sup> every antigen has five zones depending upon the dilution: (1) only negative results with all sera, (2) syphilis positive, nonsyphilitic negative, (3) syphilis and other diseases positive, normal persons negative, (4) all sera positive, and (5) self-inhibition. Only the second zone is specific for syphilis.

From this viewpoint the sixteen antigens could be divided into four groups (the Roman figures indicate the position in the sensitivity table [see Table IV]):

A. Absolutely specific were Pe 2 (I), Ac 2 (II), Ac 4 (III), 4 (XV), Pe 4 (X).

B. Slightly nonspecific were E 3 (XVI), 3 (XIII), E 1 (VI), Pe 1 (IX), and Pe 3 (XII).

C. Medium positive nonspecific reactions were shown by E 4 (XI), 1 (XIV), and 2 (VIII), with cord or uterine blood. Ac 3 (VII) reacted more with sera from patients with diseases such as pneumonia, cancer, leucemia, etc.

D. Nonspecific reactions with all types of nonsyphilitic sera were shown by Ac 1 (IV) and E 2 (V), the latter yielding the most and strongest of the nonspecific reactions.

The positive reactions with nonsyphilitic sera were usually stronger than the positive reactions with sera from syphilitic patients. It may be possible to achieve with these nonspecific antigens an inverse verification test. Further investigation on this interesting phenomenon is being carried out and a special paper on the subject will soon be published.

#### DISCUSSION

Since Wassermann, Neisser, and Bruck first published their test for syphilis, a steady development in the preparation of antigens has taken place. The watery emulsion of fine particles from syphilitic fetal livers was followed by an alcoholic extraction of the same organ. It was replaced by the whole alcoholic extraction of normal organs, usually heart muscle of beef or man. These antigens were made more sensitive by adding cholesterol. Noguchi and Bronfenbrenner<sup>11</sup> introduced the acetone-insoluble fraction of alcoholic extractions of beef heart as antigen. Similar antigens were prepared by Bordet and Ruelens,<sup>12</sup> Wadsworth,<sup>13</sup> Kolmer,<sup>14</sup> and others. Scaltritti<sup>15</sup> used an alcoholic

solution of cadmium chloride to precipitate a certain group of lipoids. Through absorption of tissue extracts on aluminum hydroxide and on rare earths, such as bentonite and floridin, Balbi<sup>16</sup> tried to obtain a more specific antigen.

From the chemical viewpoint, the most extensive investigations of "lecithin-like substances of the myocardium" were performed by Erlandsen<sup>17</sup> (1907). He recommended the abolition of the old conception of lecithin and replaced it with a new and more comprehensive one in which the phosphatides were divided into four groups:

1. Monoamido-monophosphatide; the relation between nitrogen and phosphorus is 1:1; it consists of lecithin and cephalin.
2. Monoamido-diphosphatide; the relation between nitrogen and phosphorus is 1:2; it consists of cuorin.
3. Diamido-monophosphatide; the relation between nitrogen and phosphorus is 2:1; it consists of amidomyelin and sphingomyelin.
4. Diamido-diphosphatide; the relation between nitrogen and phosphorus is 2:2; it does not exist in the myocardium.

Ten years later (1917) Neymann and Gager<sup>18</sup> published a new method for making Wassermann antigens from normal heart tissue which was partly based on Erlandsen's analysis of the different phosphatides. In one experiment they extracted dried pulverized beef heart with ether, acetone, and alcohol. In another experiment they used, as starting material, the secondary alcohol extraction and treated this with ether, acetone, and alcohol. They found "that lecithin is the only substance of the primary ether extract that has any antigenic value, and that the most important substance, as regards antigenic value, occurs in the secondary alcohol extract and is a diamino-monophosphatide."

Pangborn<sup>19</sup> isolated a new serologically active phosphatide from beef heart by a special method of extraction. It was named cardiolipin and is essential for the reactivity of beef heart antigens in the serologic tests for syphilis. It is important to note that it must be prepared from fresh beef heart tissue by a complicated method: precipitation by  $\text{BaCl}_2$  from methyl alcohol extracts of tissue and successive purification over barium and cadmium salts.

Few of the mentioned phospholipides can be compared with the fractions prepared by the method described in this paper. The antigens are not chemically pure but are a mixture of different phospholipids. For example, E 1 and E 2 can be compared with the B of Neymann and Gager, fats with slight amount of phosphatides and a complement fixating power of 1:1600; E 3 can be compared slightly with the A of Neymann and Gager, which has no binding power. E 4 is perhaps a combination of C (lecithin), D (cephalin), and E (cuorin). Of these, only C has the higher binding power 1:800.

Another approach to eliminate the nonspecific lipoids was made by the introduction of spirochetal antigens. Spirochetal antigens were first prepared by Hinklemann,<sup>20</sup> Gaetgens,<sup>8</sup> and Klopstock.<sup>9</sup> At the Washington Serology Conference, two serologists (Eagle and Kolmer), used spirochetal antigens, but the results were not as satisfying as expected from previous reports by other authors. In a previous publication,<sup>10a</sup> Klopstock's original spirochetal antigen was shown to be very good when it was used with heated sera.

Extraction with petrol ether was first recommended by Klosterman and Weisbach<sup>21</sup> because it extracts less of the active substances. They used it only as a preliminary extraction before making the real one and made no use of

the petrol ether-soluble lipoids. The comparison of the petrol-soluble and -insoluble lipoids shows that the soluble ones also can be used successfully for diagnosis of syphilis as shown in Tables I to IV. The slight preponderance of sensitivity of the antigen gained through a previous petrol ether extraction over the antigen gained through a previous acetone extraction means that the first-mentioned procedure gives more efficient lipoids in spite of the fact that the quantity is only about one-half (0.440 Gm.) of the amount gained by a previous acetone extraction (0.860 Gm.).

The main difference between both procedures lies in the greater amount of acetone-soluble lipoids yielded by a previous acetone extraction (1.020 Gm.) as against 0.637 Gm. with a previous petrol ether extraction. The amount of the acetone-insoluble fractions is almost identical. When the first extraction was made with acetone, 0.690 Gm. of the acetone-insoluble fractions was obtained; when petrol ether was used for the first extraction, 0.780 Gm. was gained.

#### CONCLUSION

1. The most effective fraction of the lipoids of dried beef heart is the petrol ether-soluble one.
2. The acetone-soluble fraction of petrol ether-soluble fraction is more sensitive than the acetone-insoluble fraction.
3. Whether the extraction is first made with acetone and then with petrol ether or reversed does not make too much difference, but the former procedure seems to yield a slightly superior antigen.
4. A previous ether extraction renders antigens of inferior value, the best of them (E 2 and E 1) occupying the fifth and sixth place in sensitivity and having very low specificity.
5. The petrol ether- and acetone-insoluble fractions (Pe 3 and Ac 3) can be used to verify a nonspecific positive reaction inasmuch as they give a stronger positive reaction with nonsyphilitic sera than do the "specific" antigens. Syphilitic sera either do not render a positive reaction with these antigens at all or one much weaker than the "specific" antigens.

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## A STUDY OF THE RELATIVE SENSITIVITY OF DIFFERENT LOTS OF ANTIGEN EMPLOYED IN THE SEROLOGIC TESTS FOR SYPHILIS

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THE Kahn tests have been routinely employed in the laboratories of the Georgia Department of Public Health for many years. Antigens used have been either those prepared in our laboratory and corrected to standard requirements by Kahn's laboratory or those approved by Kahn and purchased from commercial firms.

Before a new lot of antigen was placed in use, it was run in parallel with the current lot of antigen on several series of weak-reacting sera. In this way, the relative sensitivity of the two antigens in routine work could be determined. Some antigens compared quite favorably, while others showed more marked differences in sensitivity.

If the level of sensitivity had gradually been lowered or elevated by successive antigens, then a new lot of antigen, with a reverse trend, would tend to restore the sensitivity approximately to the desired level. If the sensitivity of the test continued to be lowered or elevated beyond certain expected comparable limits by a new lot of antigen, it caused concern, but the antigen was nevertheless used, since it had been approved for a satisfactory sensitivity level by Kahn's laboratory.



A Kahn antigen with an observed low sensitivity was placed in routine use and was employed in the 1944 National Serology Evaluation Study. A poor rating in sensitivity, compared to Kahn's laboratory and to other tests employed in our laboratory on these specimens, served as a stimulus for this study.

Arrangements were made with one of the local venereal disease clinics to furnish specimens of blood from patients receiving antisyphilitic treatment. Lists of names of patients who had given weak or moderate reactions by the current lot of antigen in routine use in our laboratory were furnished to the clinic at weekly intervals. The clinicians secured from these patients, upon their next visit to the clinic, ample specimens of blood and forwarded them to the laboratory.

Seven approved Kahn standard antigens were secured for this study. Sources of these antigens are listed at the conclusion of this paper.

The same technician performed all tests in parallel with all antigens. The specimens were examined in groups of five so as to conform to the time requirements in the use of the antigen emulsions. Precautions were taken to circumvent factors which might be responsible for differences in results.

Antigen suspensions were prepared and used in numerical sequence beginning with antigen 1 in the first group of specimens, antigen 2 in the second group, and so on in rotation throughout the study, so that no antigen would be given any favored position. A separate pipette was used to introduce each antigen emulsion into the test tubes.

The sera were introduced with a separate nondelivery pipette into all antigen emulsions in the same order that antigens were introduced. Each serum was allowed to stand with its antigen mixture for the same length of time, between three and ten minutes, before the three-minute shaking period. Controls on each antigen were run with each group of specimens.

The tubes for the tests were set up so that a separate rack contained the test on each serum with all the antigens. The first set of tubes in the first rack was diluted with saline and read immediately, and then the remaining sets in the numerical order of the antigens were treated in like manner. The other racks were diluted and read in sequence in the same way. A fifteen-minute reading was also made on all tests.

Some sera were found to give 3 or 4 plus reactions by all antigens. These results were excluded from the study. A quantitative test was performed on each of these sera and a dilution prepared which would apparently be critical for all antigens. The tests were repeated on these saline dilutions of sera and the results recorded.

The results on the 100 specimens examined by the Kahn standard test in this study are given in Table I, and the difference in sensitivity of the seven antigens is shown. The classification of the results from the tube readings is the same as that used for routine reporting and follows very closely the recommendation of Kahn. Sensitivity percentages are calculated on the same basis employed in the National Serology Evaluation Studies.

Dispersibility of the precipitates between the immediate and fifteen-minute readings was much more pronounced with some antigens than with others. It will be observed (Table I) that antigen 4, the most sensitive, showed the least dispersibility, a drop of 7.5 per cent in sensitivity, and that antigen 6, the next highest in sensitivity, exhibited the greatest dispersibility, a drop of 21.5 per

TABLE I. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE KAHN STANDARD TEST

ANTIGEN	1	2	3	4	5	6	7
<i>Immediate Reading</i>							
Positive	61	62	68	93	55	82	59
Doubtful	23	25	20	5	22	14	22
Negative	16	13	12	2	23	4	19
Sensitivity	72.5	74.5	78.0	95.5	66.0	89.0	70.0
<i>Fifteen-Minute Reading</i>							
Positive	39	47	51	81	25	53	36
Doubtful	32	35	33	14	43	29	30
Negative	29	18	16	5	32	18	34
Sensitivity	55.0	64.5	67.5	88.0	46.5	67.5	51.0
<i>Average of Above Readings</i>							
Positive	47	52	63	83	41	70	45
Doubtful	29	31	23	15	31	19	27
Negative	24	17	14	2	28	11	28
Sensitivity	61.5	67.5	74.5	90.5	56.5	79.5	58.5

cent. Antigens 3 and 6 showed 11 per cent difference in sensitivity in the immediate readings and yet gave identical sensitivity in the fifteen-minute readings.

Taking the average of the immediate and fifteen-minute readings as a basis for comparison, antigen 4 showed the highest sensitivity (90.5 per cent) and antigen 5 the lowest (56.5 per cent), a spread of 34 per cent between these extremes.

It was then decided to repeat this study on another group of 100 specimens with a different group of five antigens selected from the sources listed at the conclusion of this paper. Specimens were secured in the same manner as for the first study and all of the same safeguards of technique which might influence the results were observed. In this study with five antigens, the specimens were examined in groups of seven. Specimens giving strong reactions were diluted with saline to the critical point for the antigens.

The comparison of the sensitivity of the antigens in the immediate, fifteen-minute, and average readings is given in Table II. It will be noted that the dispersibility of the precipitate in the two most sensitive antigens, 10 and 12, and the least sensitive antigen, 8, was approximately the same and was much less than for the other two antigens.

Based on the average readings, antigen 10 was the most sensitive (95.5 per cent) and antigen 8 the least sensitive (39.0 per cent), a difference of 56.5 per cent between these extremes. Antigens 10 and 12 were quite comparable in sensitivity in all readings.

It was then decided to determine if this observed variation in sensitivity was peculiar to the Kahn standard antigens, or if the antigens employed in the other recognized serologic tests would exhibit similar differences in sensitivity. Antigens were secured for this part of the study from sources listed at the conclusion of this paper. It will be noted, in this connection, that one antigen was secured from the laboratory of each author-serologist.

Specimens were secured in the same manner as for the comparison of the Kahn antigens. The techniques of the authors of the tests were rigidly observed. All possible safeguards were set up to insure parallel performance in every detail. Classification of plus reactions into positive, doubtful, or negative was followed as recommended by each author.

TABLE II. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE KAHN STANDARD TEST

ANTIGEN	8	9	10	11	12
<i>Immediate Reading</i>					
Positive	28	67	96	75	92
Doubtful	43	27	1	15	5
Negative	29	6	0	10	3
Sensitivity	49.5	80.5	98.0	82.5	94.5
<i>Fifteen-Minute Reading</i>					
Positive	19	35	77	33	72
Doubtful	34	43	18	37	20
Negative	47	22	5	30	8
Sensitivity	36.0	56.5	86.0	51.5	82.0
<i>Average of Above Readings</i>					
Positive	20	52	93	61	87
Doubtful	38	34	5	26	11
Negative	42	14	2	13	2
Sensitivity	39.0	69.0	95.5	74.0	92.5

The different sensitivity levels of the various tests precluded the acceptance of the same group of specimens for all tests. While more than one test was performed on many of the specimens, they may be considered for the purpose of this study as a series of 100 different specimens for each test.

To illustrate, the Kline and Mazzini tests may have been performed on the same specimen. If the three antigens used in the Kline test gave very weak reactions and any one of three antigens used in the Mazzini test gave less than a 4 plus reaction, the specimen was accepted as critical for both tests. If any one of the antigens in the Kline test gave less than a 4 plus reaction and all three Mazzini antigens gave 4 plus reactions, the specimen was discarded from consideration in the Mazzini test series. If, on the other hand, the Mazzini antigens showed variation in results and all Kline antigens gave negative reactions, the specimen was not considered critical for the Kline test.

Specimens giving 4 plus reactions by all antigens in any given test were diluted serially and the test repeated in quantitative manner. The lowest dilution showing variation in plus reactions was accepted as the critical point for the antigens in that test.

It will be noted from Table III that Kline antigens 1 and 2 gave quite comparable results. Antigen 3 was far less sensitive.

The relative sensitivity of three antigens employed in the Eagle microflocculation test is shown in Table IV. Antigen 2 was found to be much less sensitive than either of the other two antigens.

TABLE III. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE KLINE DIAGNOSTIC TEST

ANTIGEN	1	2	3
Positive	62	61	39
Doubtful	34	33	28
Negative	4	6	33
Sensitivity	79.0	77.5	53.0

TABLE IV. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE EAGLE MICROFLOCCULATION TEST

ANTIGEN	1	2	3
Positive	67	26	55
Doubtful	28	21	34
Negative	5	53	11
Sensitivity	81.0	36.5	72.0

TABLE V. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE HINTON FLOCCULATION TEST

ANTIGEN	1	2	3
Positive	70	45	61
Doubtful	28	39	28
Negative	2	16	11
Sensitivity	84.0	64.5	75.0

TABLE VI. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE MAZZINI FLOCCULATION TEST

ANTIGEN	1	2	3
Positive	60	74	68
Doubtful	39	26	31
Negative	1	0	1
Sensitivity	79.5	87.0	83.5

It will be observed from Table V that there is a difference of 19.5 per cent in the sensitivity of antigen 1 and antigen 2 used in the Hinton test.

From Table VI it may be seen that the antigens used in the Mazzini test compared rather favorably in sensitivity.

In the Kolmer test, it was more difficult to secure specimens which would reflect marked differences in sensitivity based on positive, doubtful, and negative results as classified by Kolmer. For instance, a specimen giving 1 plus, 2 plus, and 4 plus reactions by three different antigens would be recorded as positive by all three antigens.

Therefore, in order to maintain a sensitivity level of the Kolmer test in a relative position with the other serologic tests, it was necessary to incorporate more of the type of specimens which gave very weak reactions.

It will be observed from Table VII that antigens 1 and 3 in the Kolmer test compared very favorably in sensitivity. Antigen 2 was found to be much less sensitive than the other two antigens.

TABLE VII. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE KOLMER SIMPLIFIED TEST

ANTIGEN	1	2	3
Positive	69	46	69
Doubtful	27	14	25
Negative	4	40	6
Sensitivity	82.5	53.0	81.5

#### COMMENTS

This study is admitted to be rather critical in that it includes an unusually large number of doubtful reactions which magnify differences in sensitivity. In routine work such differences are diluted with large numbers of definitely positive or negative reactions.

Nevertheless, serology evaluation studies incorporate a comparatively large number of weak-reacting specimens, and acceptable performance is determined by the results reported. This study seeks to show that failure to achieve satisfactory ratings in sensitivity may lie in the antigens employed and may not always reflect a lack of skill in performance.

As one lot of antigen is exhausted and another placed in use, laboratories should be able to maintain approximately the same level of sensitivity. Otherwise, specimens for recheck purpose, which give differences in results, may

lead to disconcerting situations. Particularly this may be reflected in quantitative tests.

From the rather wide variation observed in the sensitivity of some of the antigens employed in this study, it appears that more attention should be given to the standardization of this reagent. This could probably be better accomplished by the establishment of a special laboratory under some agency for the production, standardization, and distribution of all antigens employed in the various recognized serologic tests for syphilis.

#### SUMMARY AND CONCLUSIONS

Three or more different lots of approved antigens were run in parallel in each of the following serologic tests for syphilis: Kahn standard, Kline diagnostic, Eagle microflocculation, Hinton flocculation, Mazzini flocculation, and Kolmer simplified complement fixation. Antigens were secured from various sources, including at least one from the laboratory of each author-serologist. One hundred specimens of blood for each test were secured from syphilitic donors under treatment.

The technique of each author was rigidly followed. Throughout the study, all precautions for parallel conditions were observed for each procedure. Reactions were classified as positive, doubtful, and negative as recommended by each author for his test.

Specimens giving negative reactions by all antigens in any test were excluded from consideration. Specimens giving 4 plus reactions by all antigens in a given procedure were diluted serially and re-examined in a quantitative manner. The lowest dilution giving less than a 4 plus reaction by any of the antigens was considered as critical for the antigens of that test.

Sensitivity was calculated on the same basis as adopted in the National Serology Evaluation Studies. A rather wide variation was found in the sensitivity of the antigens in all the tests with the exception of the Mazzini test. This observation in the Mazzini test should be considered as negative information, since the number of antigens employed was too small to be conclusive in this respect.

This study was more critical for the sensitivity of antigens than would be experienced in routine work. However, differences in sensitivity ratings achieved in serology evaluation studies by different laboratories employing the same test may be due to the relative sensitivity of the antigens used.

It is felt that the quality of laboratory service would be further improved if a special laboratory could be established under some agency for the preparation, standardization, and distribution of all antigens employed in the serologic tests for syphilis.

The authors wish to acknowledge the excellent cooperation of Dr. E. S. Armstrong and Mrs. Mary E. Daniel, R.N., of the Atlanta Health Center, and Dr. W. A. Mason and Carrie Lou McCarter, R.N., of Westside Health Center, in securing the specimens for this study.

#### Kahn Antigens

1. University Hospital Laboratories, Ann Arbor, Mich., Lot No. 67 A.
2. Georgia Department of Public Health, Atlanta, Ga., Lot No. K-1.
3. Georgia Department of Public Health, Atlanta, Ga., Lot No. F.
4. Digestive Ferments Co., Detroit, Mich., Lot No. 45759.
5. Digestive Ferments Co., Detroit, Mich., Lot No. 46846.
6. Digestive Ferments Co., Detroit, Mich., Lot No. 47452.
7. Alabama Department of Public Health, Montgomery, Ala., Lot No. 29 A.

8. University Hospital Laboratories, Ann Arbor, Mich., Lot No. 71.
9. Digestive Ferments Co., Detroit, Mich., Lot No. 47452.
10. V. D. Research Laboratory, Staten Island, N. Y., Lot No. 8 (1944).
11. Navy Medical Center, Washington, D. C., Lot No. 89 B.
12. Army Medical Center, Washington, D. C., Lot No. 171.

#### *Kline Antigens*

1. LaMotte Chemical Products Co., Baltimore, Md., Lot No. 241.
2. LaMotte Chemical Products Co., Baltimore, Md., Lot No. 242.
3. Dr. B. S. Kline, Cleveland, Ohio (lot number not given).

#### *Eagle Antigens*

1. Digestive Ferments Co., Detroit, Mich., Lot No. 46151.
2. District of Columbia Department of Health, Washington, D. C. (lot number not given).
3. Dr. Harry Eagle, Baltimore, Md. (lot number not given).

#### *Hinton Antigens*

1. Digestive Ferments Co., Detroit, Mich., Lot No. 44163.
2. Maryland Department of Public Health, Baltimore, Md., Lot No. 18 X.
3. Dr. William A. Hinton, Boston, Mass., Lot No. 224.

#### *Mazzini Antigens*

1. Eli Lilly and Co., Indianapolis, Ind., Lot No. 3278-366276.
2. Eli Lilly and Co., Indianapolis, Ind., Lot No. 0169-350122.
3. Mr. L. Y. Mazzini, Indianapolis, Ind. (lot number not given).

#### *Kolmer Antigens*

1. Digestive Ferments Co., Detroit, Mich., Lot No. 46071.
2. Digestive Ferments Co., Detroit, Mich., Lot No. 43450.
3. Research Institute of Cutaneous Medicine, Philadelphia, Pa. (lot number not given).

## THEORY OF THE COLLOIDAL GOLD REACTION

### I. REACTIONS BETWEEN GOLD SOL AND ISOLATED PROTEIN FRACTIONS

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SCHULZ and Zsigmondy's gold number<sup>1</sup> was the incentive which led to the development of the diagnostic gold reaction in cerebrospinal fluid.<sup>2</sup> The gold number, the minimum amount of a protein (colloid) that protects 10 c.c. of formol gold against coagulation by 1 c.c. of 10 per cent saline, had been used only for characterizing isolated protein fractions and not for mixtures such as occur in cerebrospinal fluids. Application of the gold number to such specimens proved to be of minor significance.

In studying syphilitic cerebrospinal fluids, it was suspected that syphilis reagin, being an abnormal euglobulin fraction, was insoluble in the distilled water used as diluent in the gold number. Therefore, cerebrospinal fluids were serially diluted with 0.4 per cent saline before the formol gold was added. Various coagulation curves instead of the expected protection were obtained. The theoretical significance of the results was not realized at first. Interest was focused on the practical discovery that these curves seemed to be of clinical

TABLE I. REACTIONS WITH PROTEIN FRACTIONS AND THEIR MIXTURES

SERIAL NUMBER	MATERIAL	SERIAL DILUTIONS IN STRONG BUFFERS <sup>1</sup>														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	20 mg. per 100 c.c. albumin	1:1.5	2:1	3:1	4:1	5:1	6:1	7:1	8:1	9:1	10:1	11:1	12:1	13:1	14:1	15:1
2	20 mg. per 100 c.c. globulin	0	1	1.5	2	3	4	5	6	7	8	9	10	11	12	13
3	5 mg. per 100 c.c. pseudoglobulin	0.5	10	16	15	13	12	8.5	7.5	5	4	3.5	2.5	1.5	1	1
4	20 mg. per 100 c.c. pseudoglobulin	11	16	17	18	17	9	6.5	5.5	5	3.5	2	1.5	1	1	1
5	100 mg. per 100 c.c. pseudoglobulin	2.5	3.5	7	10	13	16	14	10	7.5	5.5	5	3	1.5	1	1
6	1,000 mg. per 100 c.c. pseudoglobulin	1	1	1	1.5	2	3	5.5	9	13	15	16	15	8.5	7.5	7
	ALBUMIN- GLOBULIN IN MG. PER 100 C.C.	1	1	1	1	1	1	1	1	1	1.5	2	3	7	9.5	14
7	1,000	1	1	1	1	1	1	1	1	1	1	1	2	3	6	10
8	300	1	1	1	1	1	1	1.5	1.5	2.5	4.5	7	13	16	15	13
9	100	1	1	1	1.5	1.5	2.5	4	6.5	9	10	13	10	9	7.5	6
10	30	1.5	1.5	2	3	5	7.5	10	13	11	9.5	7	5.5	4.5	4.5	3.5
11	10	1.5	2	3.5	5	6.5	8	7.5	6.5	6.5	5.5	4.5	4.5	3.5	3	2
12	5	1.5	2	3	4.5	6	7.5	7.5	5.5	5	4.5	4.5	2.5	2	2	1.5
13	2	1.5	1.5	2	3	2.5	2.5	2.5	2	2	1.5	1.5	1	1	1	1

The numerical values are obtained with the gold color standard and express the degree of coagulation of the gold sol. 0 corresponds to the unchanged red; 20 to colorless or complete coagulation; 10, to blue; 1-9, to red-blue; and 11-19, to blue-white.

Previous experiments<sup>4</sup> proved the paretic curve to be independent of a definite albumin-globulin ratio. The question remained as to how far the ratio might be responsible for other types of curves, the prezone curves. The hypothesis that the albumin-globulin ratio is of decisive significance to the gold reaction of course implies that different globulin fractions react identically with gold sol.

In Table I, No. 2 and No. 4, are shown that identical concentrations of euglobulin and pseudoglobulin prepared from horse serum with ammonium sulfate yield characteristically different prezone curves. These simple experiments disprove the hypothesis that the albumin-globulin ratio is the only factor influencing gold reactions but fail to show what lesser role it may play. In testing various preparations of pseudoglobulin, it was learned that about 0.12 mg. per 100 c.c. gave maximal coagulation. The serial dilution of euglobulin was invariably higher, about 0.39 mg. per 100 c.c. In view of the incomplete purification obtained with salting-out procedures, it is not claimed that these numerical values are definitely established but only that euglobulin and pseudoglobulin give different quantitative reactions with gold sol.

If the albumin-globulin ratio is not the decisive factor, what does determine the location of the maximum in prezone curves? The problem has been investigated by testing isolated globulin fractions of known concentration in the presence and absence of albumins.

When pseudoglobulin solutions of increasing concentration have been tested (Table I, Nos. 3 to 6) as if they were cerebrospinal fluids of unknown constitution, the maximum has shifted increasingly to the right. In these quantitative experiments, the apparent shift to the right in the absence of albumins appears to be an expression of a *constant optimal ratio*, the maximum obtaining in those dilutions containing 0.12 mg. per 100 c.c. In other words, by testing globulin solutions of known constitution and concentration, the location of the maximum can be predicted. Inversely, when cerebrospinal fluids of unknown globulin content were tested, the maximum indicated the absolute "globulin" concentration. Although euglobulin and pseudoglobulin react differently, in mixtures such as occur in cerebrospinal fluids, their partial curves are superimposed so that the combination curve indicates only the effect of globulins as a group. The specificity of globulin fractions cannot be directly determined in the routine test.

The effect of albumins, or of the albumin-globulin ratio, upon the location of the maximum may be studied in two ways: by testing artificial mixtures of globulins and albumins and by testing cerebrospinal fluid before and after the removal of albumins.

Only negative results were obtained with artificial mixtures of euglobulins, pseudoglobulins, and albumins in attempting to produce a true paretic curve.<sup>4, 5</sup> A plateau curve resulted only in the presence of a *true coagulator*. The claimed parallelism between increasing albumin-globulin ratio and shift to the right<sup>6</sup> was based on experiments requiring albumins completely free of globulins, a precaution technically difficult to observe. We have measured the effect of an increasing albumin-globulin ratio by testing a globulin solution of known concentration, which located the maximum in one of the serial dilutions, and then adding increasing amounts of albumin. It was observed that the maximum shifted to the right.

In repeating these experiments, it was noted that the shift to the right produced by the admixture of albumins was widely different when various albumin preparations were used. Reznikoff<sup>7</sup> described the effect of albumin upon gold sol as producing complete coagulation over a wide range. Other authors have found the effect to be exclusively or predominantly protective.<sup>8</sup>



As has been mentioned, albumins exhibit decreasing coagulation when progressively purified by salting-out methods. This suggests that albumins which coagulate strongly have probably been contaminated with globulins. The same explanation was found to apply in our initial experiments, which inadvertently produced increasing concentration of globulins and thus caused a shift.

The use of protein fractions purified by electrophoresis might have been of interest. However, because all the previous experiments were performed with fractions separated with the conventional ammonium sulfate concentrations, the same procedure had to be employed. It was found that the use of albumin (horse serum), after repeated reprecipitation by 60 per cent saturation with ammonium sulfate, supplied definite results if the globulin rather than the albumin was varied. In Table I, Nos. 7 to 13, are shown the results of such experiments. If these results are interpreted by previous standards, the explanation would be that the maximum shifts to the left rather than to the right with increasing albumin-globulin ratio. Actually, these experiments prove that the changing location of the maximum has nothing to do with the albumin-globulin ratio. The maximum is exclusively determined by the absolute concentration of the globulins. The optimal ratio of 0.12 mg. per 100 c.c. remains constant in the presence or absence of albumins. Deviations from this constant optimal ratio appear only where comparatively low globulin concentrations are used (Table I, Nos. 11 to 13). The contamination of the albumins has a marked effect on the actual globulin concentration.

The real effect of albumin in albumin-globulin mixtures seems clearly demonstrated by these experiments, for in comparing identical globulin concentrations in the presence and absence of albumins, it appears that the maximum remains the same; only the strength of coagulation in the maximum is reduced as the albumin admixture increases.

These results suggest that the coagulation curves observed in the gold reaction are affected by the presence of albumins, or the albumin-globulin ratio, only to the degree that the strength of coagulation may be reduced by protecting albumins. This explanation of the relatively subordinate role of the albumin-globulin ratio applies also to the results obtained in cerebrospinal fluids as demonstrated by the following experience. Cerebrospinal fluids of high protein concentration were used. One specimen was from a patient with paresis and another from one with tuberculous meningitis; in the latter the protein concentration was approximately 150 mg. per 100 c.c. The precipitate obtained with 50 per cent saturation of ammonium sulfate was separated by centrifuging in an angle centrifuge and dialyzed against phosphate buffer of pH 7.4 used in the routine technique and diluted to the original volume. The only difference in gold curve due to the removal of the albumin was an increase in the strength of the reaction, the plateau of the paretic curve being slightly longer. The prezone curve obtained with the tuberculous cerebrospinal fluid was qualitatively the same, and only the strength of coagulation in the maximum was increased. In short, the results of the experiments with artificial mixtures seemed to be applicable to those obtained with cerebrospinal fluids, both in the paretic and prezone curves, indicating that the qualitative differences in the gold curves was unrelated to the albumin-globulin ratio.

#### SUMMARY AND CONCLUSIONS

The colloidal gold reaction has been studied by analyzing the physicochemical factors of the milieu and by testing various combinations of protein fractions.

Three qualitatively different reactions with gold sol have been demonstrated:

- (1) protection is produced both by albumins and normal serum globulins, in the prezone, and by hemoglobin at a pH above its isoelectric point; (2) sensitization is produced by serum globulin within an optimal range, electrolytes being required to complete the coagulation; (3) true coagulation, without the assistance of electrolytes, is produced in cerebrospinal fluid only by the pseudoglobulin-like *degenerative protein* indicative of parenchymatous degeneration which elicits the so-called paretic or plateau curve.

The plateau curve is fundamentally different from the remaining types, the prezone curves. The prezone curves in the absence of a *true coagulator* result from the qualitative and quantitative differences of the globulins, while the albumins, as protectors, have only a quantitatively decreasing effect. Euglobulin and pseudoglobulin react differently with gold sol; they may be characterized by the difference of their constant optimal ratio. This constant optimal ratio is the reason why, in prezone curves, the maximum shifts to the right with increasing globulin concentration.

Our interpretation ascribes to the albumin-globulin ratio the least important role in producing the qualitative differences of the various types. Since, according to a widely adopted hypothesis, the albumin-globulin ratio is the only factor producing these differences, the experimental data that are at variance with this assumption may be briefly summarized:

1. No mixture of globulins and albumins, no variation of the albumin-globulin ratio, is able to produce a plateau curve in the absence of a *true coagulator*.

2. Euglobulin and pseudoglobulin exhibit characteristically different optimal ratios.

3. The maximum does not shift to the right with increasing albumin-globulin ratio, as inferred from experiments in which albumins contaminated with globulins were used. The location of the maximum is entirely independent of the albumin-globulin ratio; it is regulated by qualitative and quantitative differences of the globulins.

4. The removal of albumins from cerebrospinal fluids, so that any effect of the albumin-globulin ratio is eliminated, produces no qualitative change either in the paretic curve or in the prezone curves.

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## EXPERIMENTAL PRODUCTION OF PULMONARY EMBOLISM BY THE USE OF A VENOUS CATHETER

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MANY methods have been devised for the experimental production of pulmonary embolism. These methods readily fall into two groups, embolism and ligation. The method most commonly employed has been the introduction of various kinds of foreign bodies into the peripheral venous system. These have consisted of such miscellaneous substances as suspensions of barium sulfate, Prussian blue, and powdered charcoal, as well as larger particles such as blood clots, masses of fibrin, beads of enamel, lead shot, rubber finger cots, mercury, waxes, and many others. The choice of the size of the particles of the embolic material used has depended upon the size of the pulmonary vessels which the investigator has wished to occlude. Less frequently, various types of ligatures and clamps have been used to occlude partially or completely the lumen of the main pulmonary artery or its branches. Haggart and Walker<sup>1</sup> devised an ingenious method of clamping the pulmonary artery which enabled them to make a quantitative estimation of the degree of obstruction. The literature of experimental embolism has been reviewed by Bardin<sup>2</sup> (1934) and by Currens and Barnes<sup>3</sup> (1943).

Both methods have distinct and obvious limitations. The site of lodgment of the embolus cannot be adequately controlled once the mass has been introduced into the systemic vein, and its ultimate action and effect on the lung cannot be predicted with precision. Direct ligation has the disadvantage that the thorax must be opened with its consequent disturbances of pulmonary and cardiac dynamics, and usually the animal must be sacrificed immediately following the experiment.

The present communication is a description of a technique for the production of pulmonary embolism which appears to offer many advantages over hitherto published methods.

### METHOD

Dogs were anesthetized by injecting nembutal (40 mg. per kilogram) intraperitoneally. The neck was shaved and one of the external jugular veins was exposed. Venous catheterization was performed by the method of Forsman<sup>4</sup> and Courmand and Ranges.<sup>5</sup> The animal was placed on its back on a fluoroscope table, and a No. 8 or No. 12 catheter, 60 cm. long with the tip bent at an angle of about 60 degrees, was inserted into the jugular vein. A slow drip of normal saline was maintained through the catheter to prevent clotting. Under direct fluoroscopic examination, the catheter was directed down the jugular vein through the superior vena cava and into the right auricle. The tip of the catheter was then turned posteriorly and to the animal's left and

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directed through the tricuspid valve into the right ventricle. Further manipulation of the catheter with the curved tip in an upward direction resulted in the catheter being deflected from the opposite wall of the ventricle upward through the pulmonary valve and into the pulmonary artery. Once in the pulmonary artery it was possible to direct it into the right or left branch and into the smaller ramifications if so desired. Its position in a pulmonary artery may be ascertained with certainty by observing the tip of the catheter to lie outside the cardiac shadow when viewed in an anteroposterior or lateral position (see Fig. 1).



Fig. 1.—X-ray of a dog with catheter introduced through the jugular vein, superior vena cava, right auricle, right ventricle, and pulmonary artery. The tip of the catheter lies in a branch of the right pulmonary artery. Left, anteroposterior view: right, right lateral view.

With the catheter in the desired location, emboli may be injected directly into the pulmonary circulation. In this study, Japan wax has served as a useful medium for studying the location of the embolus in the lung because its melting point is low ( $40^{\circ}\text{C.}$ ) and it readily congeals at body temperature of the dog. Other embolic agents may be used with equal facility. An embolic substance with a high melting point cannot be used effectively due to the marked heat loss as the substance passes through the catheter with consequent plugging of the catheter. If such a substance is heated to a point which will offset the plugging of the catheter, it will be so hot when it reaches the lung that tissue destruction may occur. The addition of methylene blue or some other vivid coloring agent to the embolic material aids in its identification in the lung.

Because the thermal death point of tissue is approximately  $47^{\circ}\text{C.}$ , care must be taken that any embolic material reaching the pulmonary circulation is below this temperature. In order to determine the degree of heat loss of the wax as it passed through the catheter, the following procedure was carried out. The catheters were immersed in a constant temperature water bath set at  $40^{\circ}\text{C.}$  which was continuously agitated. It was found that when the wax was heated to  $45^{\circ}\text{C.}$  and injected rapidly through a No. 12 catheter, the temperature of

the wax coming from the tip of the catheter was from 43 to 44° C. When the wax was heated to 85° C. and injected rapidly through a No. 8 catheter under similar conditions, the temperature of the effluent was from 45 to 46° C. It is assumed, therefore, that the temperature of the wax as it entered the pulmonary circulation was at approximately the same temperature.

The extent of the pulmonary vascular bed deprived of its blood supply can be readily controlled by the amount and type of embolic material used. In dogs weighing from 10 to 20 kilograms, 10 c.c. of Japan wax were sufficient to fill a lobular artery and cause the animal to die in from five to fifteen minutes. One animal, receiving only 3 c.c. of wax in a branch of the pulmonary artery supplying the right lower lobe was still alive one hour later and was killed by pithing.

An example of the type of embolism obtained by the use of Japan wax is shown in Fig. 2. It should be noted that the embolus is in the form of a cast of the pulmonary artery and is quite reminiscent of emboli seen in massive pulmonary embolism in man.



Fig. 2.—The arrow points to the wax cast of the pulmonary artery. Note the close resemblance to emboli found in pulmonary arteries in the human being.

The endothelium lining the jugular vein at the site of catheterization and that lining the vena cava, right auricle, right ventricle, and pulmonary arteries was carefully examined in eight dogs to determine if the catheter had traumatized the endothelium. The valves and papillary muscles were also examined. In no instance was there any evidence of damage to any of these structures.

#### DISCUSSION

We believe that the present method has definite advantages over those hitherto described. An embolus of any desired character may be introduced into any part of the pulmonary circulation at a given instant. By this technique it is not necessary to open the chest, thereby avoiding complicating disturbances of respiratory and circulatory dynamics.

Studies of the sequence of the pathologic physiology of pulmonary embolism are being carried out by the use of this general method.

#### SUMMARY

1. A method is described by which pulmonary embolism may be produced in animals in a controllable and localized area of the lung.

2. The method consists of introducing a catheter through the jugular vein and chambers of the right side of the heart into the main pulmonary artery or one of its smaller branches. The embolic substance is injected through the catheter.

3. It is felt that by the use of this method pulmonary emboli closely simulating those found in human beings can be reproduced.

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## PENICILLIN COMBINED WITH FEVER THERAPY

### A PRELIMINARY REPORT OF TWENTY CASES OF EARLY SYPHILIS

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THE available literature offers few reports concerning penicillin-fever therapy. Barksdale<sup>1</sup> stated that penicillin combined with fever therapy was being satisfactorily used in cases of neurosyphilis. Struble and Bellows<sup>2</sup> reported on the favorable result obtained by the application of penicillin-fever therapy in a case of iridocyclitis of gonorrheal origin which had not responded to penicillin alone. Riba and co-workers<sup>3</sup> applied the cabinet type of therapeutic fever and penicillin to gonorrhea complications which had not responded to penicillin alone with encouraging results.

These data were not considered sufficient evidence that larger amounts of penicillin could be safely combined with physically induced fever or that penicillin activity could be demonstrated in the blood before, during, and after this type of therapeutic fever.

Physically induced fever combined with arseno-bismuth preparations has been employed by us in the intensive treatment of early syphilis<sup>4, 5</sup> and satisfac-

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tory clinical and serologic results have been obtained. It was observed that by increasing the amount of these chemotherapeutic agents, fewer treatment failures occurred. Toxic reactions, however, were in direct proportion to the amounts of arsenical and/or bismuth<sup>8</sup> used in combination with therapeutic fever at the 106.0° F. (rectal) level.

It was natural, therefore, to combine penicillin with this form of fever therapy, substituting a chemotherapeutic agent whose toxicity has been negligible<sup>8,9</sup> as compared to arseno-bismuth preparations which have certain limitations when administered alone in large doses or in combination with therapeutic fever.

Twenty patients having primary or secondary syphilis (dark-field positive) were given penicillin-fever therapy. The amount of fever for each patient was three hours at 106.0° F. (rectal) level, produced in an air-conditioned cabinet (hyperthermia). The sodium salt of penicillin was administered in the prefever period and during the course of the therapeutic fever. The patients all received a thorough diagnostic medical survey and the routine prefever preparation and postfever care.<sup>10</sup>

The first five patients received 25,000 units of penicillin intramuscularly by syringe every two hours during the twenty-four hours of the prefever period, a total of 300,000 units. The last dose was administered at 6:00 A.M. and the patients were in the fever cabinets at 8:15 A.M., reaching the 106.0° F. (rectal) level at approximately 9:15 A.M. Penicillin was administered intravenously by syringe in 100,000 unit doses at this time and again at the beginning of the second and third hours of the maintained fever. The amount of penicillin administered during fever therapy was 300,000 units, and the total amount for each patient was 600,000 units.

A second group of five patients was given the same routine treatment except that the penicillin was administered intramuscularly by syringe in 50,000 unit doses every four hours in the twenty-four hour prefever period.

Three of these ten patients had short periods of temperature elevation within the range of from 100.4 to 104.2° F. during the prefever penicillin administration. They were not ill and this was considered to be the Herxheimer reaction. During fever therapy nine of the patients showed no reactions. One patient (No. 6848) who had previously denied any drug addiction suddenly demanded a narcotic and became uncooperative when this was refused (he later admitted addiction); his treatment was discontinued for this reason after one and three-quarter hours of therapeutic fever. During the three postfever days all of the patients were ambulatory and in good condition.

In view of these favorable observations, it was decided to increase the total amount of penicillin to 1.2 million units for each of ten additional patients, 600,000 units to be administered in the twenty-four hour prefever period and 600,000 during fever therapy.

The patients were given the same routine fever therapy as was previously described. Penicillin was administered intramuscularly by syringe in 50,000 unit dosages every two hours in the twenty-four hours of the prefever period and 200,000 units intravenously by syringe at the height of fever and at the beginning of the second and third hours of maintained fever.

During the prefever penicillin administration one patient had a short period of temperature elevation to 100.2 and another to 101.8° F. These patients were not ill and this was interpreted to be the Herxheimer reaction. There were no reactions during fever therapy and the patients were alert and ambulatory during the postfever days.

In comparing the twenty patients treated with penicillin-fever therapy to patients receiving arsenicals, bismuth, and fever therapy, it was observed that during the fever, minor reactions were not evident and the patients' water balance was much less disturbed, and during the postfever period there was an absence of lethargy and fatigue with the penicillin-fever combination.

Blood penicillin determinations were made on twelve patients by the method described by Heilman and Herrell.<sup>11</sup> Blood specimens<sup>8</sup> were obtained from the antecubital vein after twelve and twenty-four hours of prefever penicillin administration, at the beginning of the maintenance period, and after one and two hours of maintained fever immediately before the intravenous penicillin was administered. Specimens were also obtained at approximately 8:00 A.M. on the first two consecutive postfever days. It is shown in Table I that penicillin activity in the blood is present during fever therapy and that there is marked variation in the values secured.

The clinical results obtained by these therapeutic methods have been of interest to us and have formed the foundation for further study of the combination of fever and penicillin in the treatment of early syphilis. The immediate clinical response to these three schedules of treatment was the same as evidenced

TABLE I. PENICILLIN LEVELS (O.U. OF ANTIBACTERIAL ACTIVITY PER CUBIC CENTIMETER OF BLOOD)

PATIENT	PREFEVER			FEVER				POSTFEVER	
	INTRAMUSCULAR SODIUM PENICILLIN ADMINISTRATION	12 HR. AFTER INITIATION OF PENICILLIN	24 HR. AFTER INITIATION OF PENICILLIN	INTRAVENOUS SODIUM PENICILLIN ADMINISTRATION	BEGINNING OF MAINTENANCE PERIOD	AFTER ONE HOUR	AFTER TWO HOURS	8:00 A.M. FIRST DAY POST-FEVER	8:00 A.M. SECOND DAY POST-FEVER
5212	25,000 O.U. q. 2 h. in 24 hr. prefever	0.48	0.06	100,000 O.U. after each blood specimen	None	0.24	0.48	None	None
6643	25,000 O.U. q. 2 h. in 24 hr. prefever	0.24	0.06	100,000 O.U. after each blood specimen	0.06	15.36	0.48	None	None
6654	25,000 O.U. q. 2 h. in 24 hr. prefever	0.48	0.06	100,000 O.U. after each blood specimen	0.06	30.00	15.36	None	None
6787	25,000 O.U. q. 2 h. in 24 hr. prefever	0.06	0.06	100,000 O.U. after each blood specimen	None	0.24	0.48	None	None
6801	25,000 O.U. q. 2 h. in 24 hr. prefever	0.48	0.03	100,000 O.U. after each blood specimen	None	0.24	0.48	None	None
6826	50,000 O.U. q. 4 h. in 24 hr. prefever	0.03	0.03	100,000 O.U. after each blood specimen	None	0.96	0.96	None	None
6830	50,000 O.U. q. 4 h. in 24 hr. prefever	0.24	0.03	100,000 O.U. after each blood specimen	None	0.48	0.96	None	None
6839	50,000 O.U. q. 4 h. in 24 hr. prefever	0.24	0.06	100,000 O.U. after each blood specimen	None	0.12	0.24	None	None
6848	50,000 O.U. q. 4 h. in 24 hr. prefever	0.48	0.03	100,000 O.U. after each blood specimen	None	15.36	0.48	None	None
6995	50,000 O.U. q. 4 h. in 24 hr. prefever	None	0.12	100,000 O.U. after each blood specimen	Trace	0.96	1.92	None	None
7523	50,000 O.U. q. 2 h. in 24 hr. prefever	0.24	0.24	200,000 O.U. after each blood specimen	0.03	0.96	1.92	None	None
7946	50,000 O.U. q. 2 h. in 24 hr. prefever	0.48	0.12	200,000 O.U. after each blood specimen	None	0.48	0.48	None	None

\*The blood penicillin specimens were sent by air mail to the Mayo Clinic and the determinations performed under the direction of Dr. Paul O'Leary, Section on Dermatology, and Dr. D. H. Heilman, Division of Clinical Pathology, to whom we are indebted.



by any form of intensive treatment of syphilis. The lesions became negative for the *Treponema pallidum* on dark-field microscopy and healed. This will occur after penicillin in amounts of 60,000 units<sup>12</sup> is administered and should be expected after the amounts used in this study.

There are no reports to our knowledge on the treatment of early syphilis using 600,000 or 1.2 million units of penicillin within a period of forty-eight hours. Such amounts of penicillin have usually been administered over a period of seven and one-half days. The immediate clinical results with such schedules have been the same as experienced in this group of patients. This fact allows us only one method of comparison and that is the relapse rate.

The indicated eventual relapse rate using 600,000 units of penicillin over seven and one-half days is 40 per cent.<sup>13</sup> The ten patients treated with 600,000 units of penicillin in two days and three hours of fever at 106° F. (rectal) exhibited a relapse rate of 80 per cent. Although the group is small, the failure of eight of ten patients is certainly significant; all the failures occurred within the first six months after treatment, further proof of the inadequacy of this treatment schedule. The results in this group are shown in Table II.

TABLE II. NUMBER OF PATIENTS WITH SYPHILIS TREATED WITH FEVER AND 600,000 UNITS OF PENICILLIN FROM JULY 26 THROUGH AUG. 24, 1944, GROUPED BY FINAL DIAGNOSIS AND LAST REPORTED STATUS PRIOR TO JULY 1, 1945

LAST REPORTED STATUS	TOTAL	DIAGNOSIS	
		PRIMARY SROPOSITIVE	SECONDARY
Total	10	2	8
Negative	1	1	-
Positive—1 to 4 units	-	-	-
Positive—Over 4 units	1	-	1
Failures	8	1	7
Total		8	
Clinical relapse	5		
Clinical progression	1		
Serologic relapse	2		

The indicated eventual relapse rate using 1,200,000 units of penicillin in early syphilis is from 15 to 20 per cent.<sup>13</sup> Two of the ten patients in this study treated by the use of 1,200,000 units of penicillin and three hours of fever at 106° F. (rectal) were not seen again after dismissal from the hospital. Two of the eight patients under observation have been classified as serologic relapses. No clinical failures have appeared in this group of patients. Both serologic failures occurred in the eighth month after treatment, at least two months later than any of the failures with 600,000 units. The clinical results in the group to date are encouraging and further study of the use of 1,200,000 units of penicillin plus physically induced fever are in progress. The serologic status to date of the patients in this group is shown in Table III.

TABLE III. NUMBER OF PATIENTS WITH SYPHILIS TREATED WITH FEVER AND 1.2 MILLION UNITS OF PENICILLIN FROM OCT. 2 THROUGH NOV. 8, 1944, GROUPED BY FINAL DIAGNOSIS AND LAST REPORTED STATUS PRIOR TO JULY 1, 1945

LAST REPORTED STATUS	TOTAL	DIAGNOSIS	
		PRIMARY SERONEGATIVE	SECONDARY
Total treated	10	2	8
Not reporting	2	-	2
Total under observation	8	2	6
Negative	3	2	1
Positive—1 to 4 units	1	-	1
Positive—Over 4 units	2	-	2
Failures (serologic relapse)	2	-	2

## SUMMARY

1. Preliminary evidence is presented to demonstrate that penicillin and physically induced fever may be safely combined in the treatment of early syphilis as described.

2. Penicillin-fever therapy as compared to arseno-bismuth-fever therapy was more comfortable for the patients.

3. Blood penicillin activity during penicillin-fever therapy was variable before and during this form of therapy, while none was detected on the two consecutive mornings after fever therapy.

4. The use of 600,000 units of penicillin plus three hours of fever at 106° F. (rectal) is inadequate treatment for early syphilis.

5. The use of 1,200,000 units of penicillin plus three hours of fever at 106° F. (rectal) is encouraging enough to warrant further study of the use of penicillin-fever therapy in early syphilis.

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## PENICILLIN THERAPY IN HUMAN BARTONELLOSIS (CARRION'S DISEASE)

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THE therapeutic effectiveness of penicillin has not been established for human bartonellosis (Carrion's disease), an illness endemic in certain regions of South America, especially in Peru and Colombia. This paper describes the clinical course of two patients with bartonellosis who were treated with penicillin and records the changes which were observed in the morphology of the responsible organism and in the degree of erythrocyte parasitism.

The general aspects of Carrion's disease have been recently summarized by Rebagliati<sup>1</sup> and Weinman.<sup>2</sup> Briefly, this disease characteristically consists of two phases or stages. The first one, to which the names of Oroya fever, non-eruptive, or hematic period are often applied, consists of a febrile period accompanied by an anemia which is usually severe, by signs of rapid red cell destruction, and by evidences of erythropoietic hyperactivity. The characteristics of this hemolytic anemia have also recently been summarized.<sup>3</sup> If the patient survives, he develops, after a variable period of time, an eruption of papular and nodular lesions which may develop in the cutaneous zone or in an internal organ. This second stage is sometimes referred to as verruga peruana, histioid phase or eruptive period. Frequent clinical variations are observed in regard to the clinical aspects and severity of these two phases. The mortality is high in the first stage of this disease and has been estimated to vary between 18 and 95 per cent, with a general average of about 40 per cent;<sup>2, 4</sup> it is thought that secondary infections are in part responsible for the high degree of mortality. In the second phase of the illness, the mortality is very low.

The responsible organism is *Bartonella bacilliformis*, discovered by Barton<sup>5</sup> in 1909. This microorganism can be observed in the red blood cells of the peripheral blood during the early stage of the disease, and it is also found by histologic examination of the eruptive lesions. Transmission of human bartonellosis is effected by means of *Phlebotomus* insects of which the species *verrucarum* appears to be the one chiefly responsible.<sup>6</sup>

### CASE REPORTS

CASE 1.—A 21-year-old woman who came from a region where human bartonellosis is endemic was admitted to the Loayza Hospital (Lima) Aug. 31, 1944. Her symptoms began twenty-three days before when she suddenly developed general malaise and irregular fever. During the first few days of the illness she received quinine and atabrine because a presumptive diagnosis of malaria had been made, and six days before admission she was given a transfusion of 100 c.c. of whole blood. On admission the patient presented marked pallor with a generalized moderate icteric tinge; she was very restless, dyspneic, and appeared to be toxic. Her temperature was 39.7° C.

Physical examination revealed no significant abnormalities except for questionable splenic enlargement. An analysis of a venous blood sample made on the day of admission gave the following results: red blood cells, 780,000 per cubic millimeter; hemoglobin, 2.7 Gm. per 100 c.c.; leucocytes, 14,970 per cubic millimeter; hematocrit, 7.6 per cent; mean corpuscular volume, 97.0 cubic microns; mean corpuscular hemoglobin, 34.0 micro micro-

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This patient has been observed during the last eleven months; her condition has remained good and at no time have the eruptive lesions, characteristic of the second stage of the disease, been observed.

CASE 2.—A 21-year-old man came to our office Dec. 11, 1944. He had been living for the previous six months in an area where human bartonellosis was endemic. His illness began eight days before with headache and chills, followed by an irregular type of fever. He also complained of pain on the right side of the neck and shoulder and of an intense feeling of malaise and weakness. The only positive findings in the clinical examination were a discrete subicteric tinge, a palpable spleen, and a few enlarged, nontender glands in the neck and inguinal regions. The temperature was 38.2° C. An analysis of a capillary blood sample, obtained on the same day, gave the following results: red blood cells, 4,320,000 per cubic millimeter; leucocytes, 8,100 per cubic millimeter; leucocytic formula, 28 per cent stab neutrophils; 31 per cent segmented neutrophils; 2 per cent eosinophils; 16 per cent monocytes; and 23 per cent lymphocytes. Examination of the blood smear showed that 3.4 per cent of the red blood cells harbored *B. bacilliformis* of the bacillary type. A second blood analysis, made two days later, showed that the red blood cell count had come down to 3,630,000 per cubic millimeter; the leucocytes were 10,850 per cubic millimeter, and the percentage of red blood cells harboring *B. bacilliformis*, all of them of bacillary type, had increased to 31.3 per cent.

The administration of penicillin\* was initiated forty-eight hours after the finding of *B. bacilliformis* in the peripheral blood. A total of 300,000 units was given in 15 doses of 20,000 units each every three hours; the first one was given intravenously and the rest of them by the intramuscular route. On the second day of the treatment the fever disappeared and there was a marked improvement in the clinical condition of the patient; the degree of parasitism at this time had decreased from 31.3 per cent of red blood cells parasited with *B. bacilliformis* to 6.8 per cent (Table II). Because of difficulties in obtaining penicillin, its administration was suspended for the next two days. This omission was followed by an aggravation of the patient's condition with a reappearance of the fever and a rapid increase in the degree of parasitism, which reached the high figure of 96.5 per cent of red blood cells with parasites. A further reduction in the number of red blood cells per cubic millimeter also occurred (Table II). Penicillin was again given every three hours intramuscularly in doses of 20,000 units each, up to a total of 800,000 units. In the sample of blood taken forty-

TABLE II. OBSERVATIONS IN CASE 2—RED BLOOD CELL COUNT AND DEGREE OF PARASITISM IN RELATION TO PENICILLIN ADMINISTRATION

SAMPLE	DATE	PENICILLIN THERAPY*	RED BLOOD CELLS (MILL. PER C.M.M.)	PARASITISM† (PER CENT)
1	12/11/44	None	4.32	3.4
2	12/13/44	None	3.63	31.3
3	12/14/44	Penicillin administered	3.23	36.6
4	12/15/44	Penicillin administered	3.43	20.0
5	12/16/44	None	3.37	6.8
6	12/17/44	None	2.98	44.6
7	12/18/44	None	2.69	96.5
8	12/20/44	Penicillin administered	1.73	90.2
9	12/21/44	Penicillin administered	1.92	63.2
10	12/22/44	Penicillin administered	2.12	36.2
11	12/23/44	Penicillin administered	1.92	6.7
12	12/24/44	Penicillin administered	2.15	2.0
13	12/26/44	None	2.40	0.9
14	12/27/44	None	2.28	0
15	12/29/44	None	2.11	0
16	12/31/44	None	1.89	0
17	1/ 3/45	None	2.31	0
18	1/ 8/45	None	2.70	0
19	1/13/45	None	3.01	0
20	1/24/45	None	3.60	0
21	2/20/45	None	4.49	0
22	6/24/45	None	4.84	0
23	8/27/45	None	5.34	0

\*Penicillin was administered in two series, 300,000 Oxford units and 800,000 Oxford units total doses, respectively. The individual doses were 20,000 units every three hours. The first series started nineteen hours before sample 3; second series was begun forty-three hours before sample 8.

†Parasitism means per cent of red blood cells parasited with *B. bacilliformis*.

\*Trademark, Schenley.

three hours after the initiation of this second series of penicillin injections, a decrease in the degree of parasitism was observed. This change became more accentuated in successive days, although the rate of disappearance of the parasitism was slower than in Case 1. No bartonellae were found in the blood two days after the last dose of penicillin.

The changes in the morphologic characteristics of the microorganisms, observed during the period of penicillin therapy, were quite similar to those observed in Case 1. About twenty-four hours after its initiation the microorganism changed its shape from the bacillary to the coccoid type and presented the staining alterations already described. It was interesting to observe that after the first series of penicillin injections had been completed, simultaneous with the relapse, a large number of microorganisms assumed again the bacillary shape with well-defined borders and a uniform color. The reinstitution of penicillin therapy, which was accompanied by the reduction in the percentage of parasited red blood cells with *B. bacilliformis*, again resulted in the morphologic variations of the type already described.

This patient has been examined on three different occasions since recovery, the last one being after about eight months. He reported that about three months after leaving the hospital he noticed a single small nodular lesion in the right gluteal region; however, no signs of this eruption could be found in an examination made one month after the reported appearance of this lesion. His general condition has remained good.

#### COMMENT

The various antiparasitic, therapeutic procedures employed so far in the treatment of human bartonellosis have not proved to be satisfactory. The product Sdt 386 B, containing 18 per cent arsenic and 20 per cent antimony, which is highly effective in controlling *Haemobartonella muris* infection in rats, has been used by Manrique and Rocha<sup>7</sup> in the treatment of fourteen cases of Carrion's disease; the opinion of these authors, who concluded that the use of the drug is beneficial, is not shared by others. Quinine, atabrine, different arsenic preparations, and sulfonamide derivatives have also been used without success. Roca<sup>8</sup> has lately recommended the use of a glycerin preparation and has reported a favorable action, but his findings have not been confirmed by other workers. Immunologic therapy, using sera prepared in animals, has been recently investigated by Howe,<sup>9</sup> but the results observed do not as yet permit a definite evaluation. Whole blood transfusions have not been widely employed as a therapeutic measure; one of the patients reported in this paper received 900 c.c. of whole blood without untoward effects.

It is evident that evaluation of the effectiveness of penicillin in the treatment of human bartonellosis can be made only after a prolonged trial, especially if we take into consideration that spontaneous recovery does occur in a large number of cases, even in those patients who show a severe degree of anemia and toxicity. It must also be remembered that secondary infections develop frequently during the first stage of the illness and, in the opinion of most observers, are in part responsible for the high degree of mortality. In these complicated cases penicillin administration may not prove to be satisfactory if the organism responsible for the associated infection belongs to a type which is refractory to the action of this drug. Cachay<sup>10</sup> has recently reported the death of an infant, with a severe degree of anemia due to human bartonellosis, who was treated with large doses of penicillin during the twenty-four hours previous to his death.

The difficulties of transmitting human bartonellosis to animals decrease the possibilities of a prompt evaluation of this therapeutic problem. Ubatuba and Vieira,<sup>11</sup> in Brazil, have observed negative results in the penicillin treatment of *H. muris* infection in rats. From the standpoint of human bartonellosis, the value of this experiment is, however, limited if it is remembered that the product Sdt 386 B, with a very uncertain action on *B. bacilliformis*, is, on the contrary, highly effective in controlling *H. muris* infection in rats. Because of

the similarities which have been noted to exist between the anatomical lesions produced by *B. bacilliformis* infection and those caused by the presence of Rickettsia, it is interesting to mention that Moragues, Pinkerton, and Greiff<sup>12</sup> observed that penicillin inhibited, in vitro, the growth of Rickettsia cultures in chick embryos and also protected rats against the development of infection with this organism.

In our opinion, the most promising aspects of the results observed in the two cases here reported are the decrease in the percentage of parasited red blood cells with *B. bacilliformis* and the very definite morphologic changes which took place in these microorganisms, carried by circulating red blood cells, within the first twenty-four hours after penicillin administration. Such changes, when found in untreated patients who recover spontaneously, do take place in a longer time and at a slower rate. Delgado<sup>13</sup> has recently observed a rapid decrease in the degree of parasitism and in the morphologic characteristics of the *B. bacilliformis* (changes similar to those described by us) in a patient with bartonellosis with a severe degree of anemia, who recovered after receiving large doses of penicillin.

It will be important to study in the future the effect of penicillin on the development of the eruptive lesions of human bartonellosis; Vila Acuña<sup>14</sup> has reported a prompt regression of cutaneous nodules in a patient treated with this drug. It will also be necessary to determine whether these lesions fail to develop in patients who have received penicillin therapy during the first stage of the illness; such occurrence, noted at least in one of our two patients, would favor the interpretation that sterilization of *B. bacilliformis* is accomplished during the period of therapy. Aldana,<sup>15</sup> who is now studying the effect of penicillin in vitro, is of the opinion that this drug has a bacteriolytic effect upon cultures of *B. bacilliformis*.

#### SUMMARY

Penicillin was employed in the treatment of two patients with bartonellosis (Carrion's disease) during the initial noneruptive period of the illness.

The favorable effects observed clinically in these patients and in the degree of red blood cell parasitism, together with the changes in the morphologic characteristics of the responsible organism in the red blood cells, suggest the desirability of carrying out further studies to elucidate the value of penicillin in the treatment of human bartonellosis.

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# A STANDARDIZED BACK BURN PROCEDURE FOR THE WHITE RAT SUITABLE FOR THE STUDY OF THE EFFECTS OF THERAPEUTIC AND TOXIC AGENTS ON LONG-TERM SURVIVAL

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**I**N ORDER to evaluate various methods of therapy in the treatment of severe thermal burns in rats, as evidenced by long-term survival, a standard burn procedure has been developed with the following objectives: (1) reproducible survival rates; (2) preservation of uninjured extremities, thus allowing the animal greater freedom of movement and the investigator untraumatized veins for infusion and hematocrit sampling; (3) elimination of complications from additional trauma and hemorrhage resulting from autoingestion; and (4) preservation of uninjured body orifices to permit voiding of urine and defecation.

*The Experimental Animal.*—The Wistar rat\* was used as the test animal. All rats received from the Wistar Institute were kept in our stock room for at least one week prior to their use. The animals were kept in small cages (five per cage) and all were fed Purina dog checkers supplemented with whole milk. Approximately twenty-four hours prior to an experiment, the desired number of rats of the proper weight range was selected at random from the stock cages and moved into the experimental room. They were kept in individual wire cages at an average room temperature of 27° C. (variation 26 to 30° C.) and an average relative humidity of 52 per cent for the duration of the experiment. Diurnal variations in temperature fell within the above range; however, because of lack of air-conditioning facilities, diurnal humidity variation was uncontrolled. This temperature was found by Horst, Mendel, and Benedict<sup>1</sup> to be that at which the minimum metabolic rate or a state of thermal equilibrium prevails. The rats had free access to food and water until about one hour prior to burning, at which time they were reweighed and carefully examined for any signs of respiratory infection. All animals exhibiting a questionable infection were discarded.

Observations on several groups of burned rats having free access to food and water showed great individual variation with respect to water intake during the first twelve hours after the burn. During this interval some rats did not drink at all. During the second twelve-hour interval following burning all rats drank some water. Hence, in order to lessen any probable effect resulting from the individual variation, water was withheld from all burned rats for the first twelve hours following the burn. Since very few animals showed any interest in food during the first twenty-four hours postburn, food was therefore withheld during this period.

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\*For reasons of availability, the rats used in these experiments were those not used for breeding because of their failure to conform to growth rate standards. The rats were otherwise healthy animals.

*Technique and Conditions of the Burn Procedure.*—A scald burn of the back was produced by immersion in a constant temperature bath (Fig. 1). The unshaved rats were anesthetized in a glass specimen jar, volume 8,816 c.c., containing a coarse mesh wire screen platform elevated 9.0 cm. over a pad of ether-saturated cotton (Fig. 1). Optimum anesthetic conditions were obtained

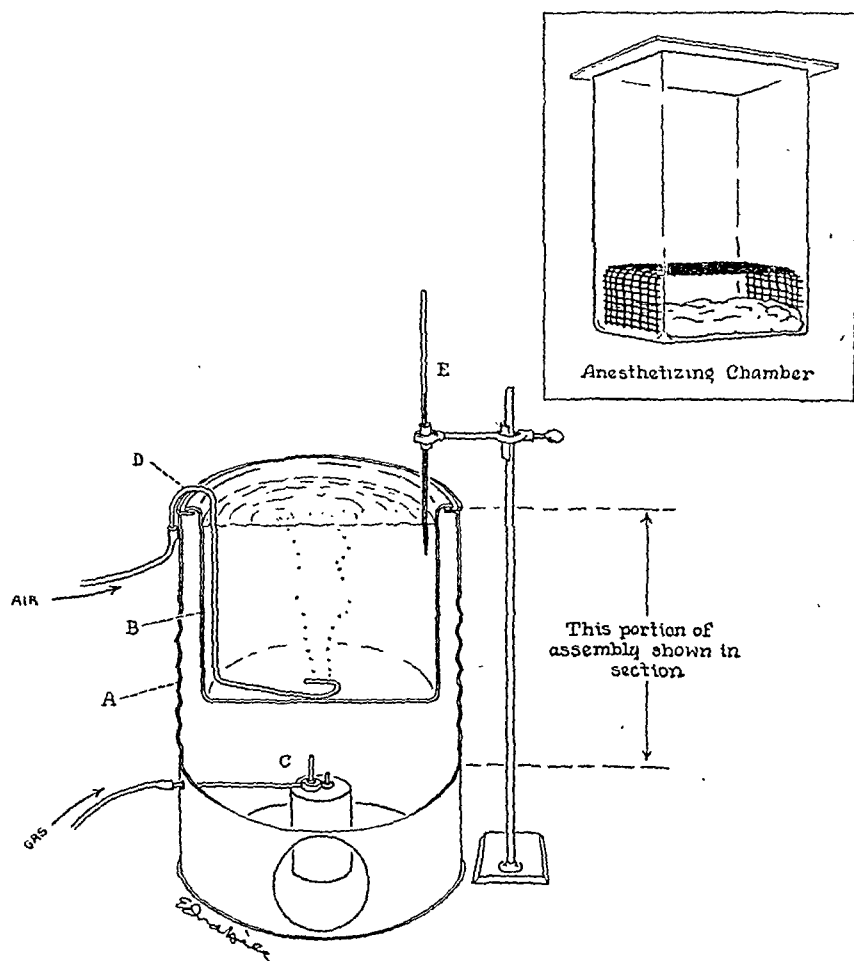


Fig. 1.

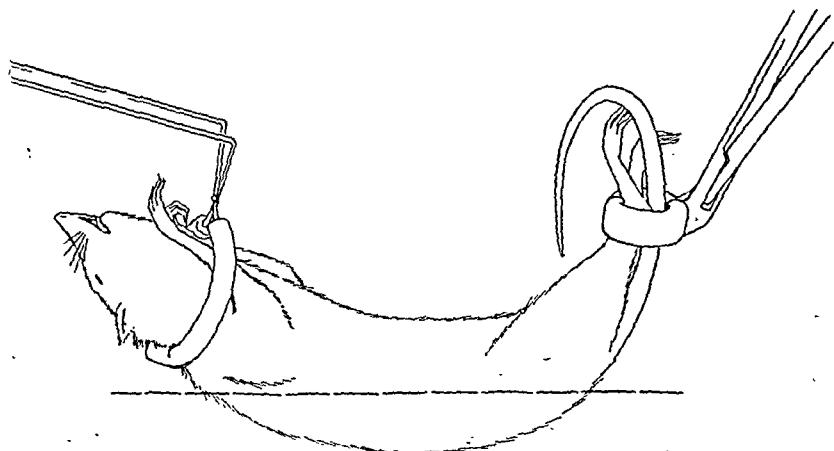
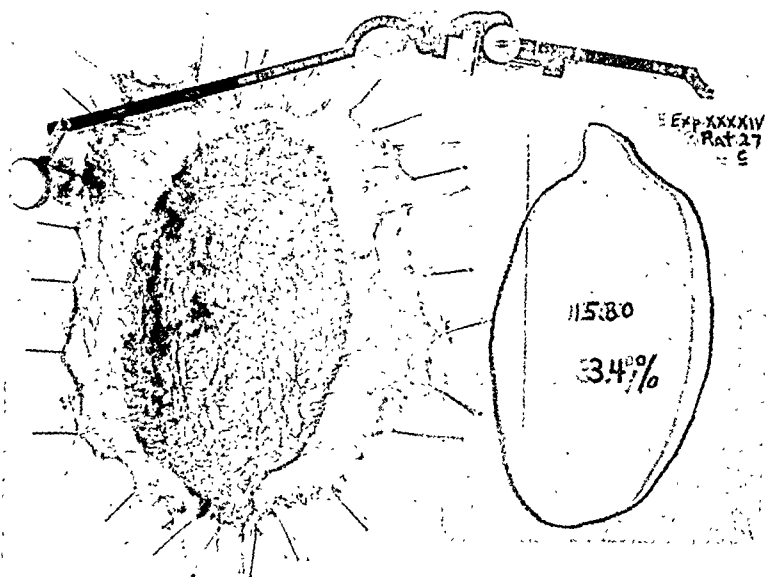


Fig. 2.



when the ether concentration was such as to produce complete anesthesia within 90 to 120 seconds. The forefeet (bound together by a rubber band) and head were held in a bone forceps (Fig. 2). The rats were immersed for fifteen seconds, timed by a stopwatch, at a bath temperature of  $90^{\circ}\text{C} \pm 0.25^{\circ}\text{C}$ . The bath temperature was recorded by a mercury thermometer lowered to a depth of 5.0 cm. below the surface of the bath. The water was continuously agitated by a compressed air bubbler placed on the bottom of the tank (Fig. 1). When removed from the bath, the rats were gently blotted by rolling them in an absorbent towel and then removed to individual wire cages.



24 hr. death  
postburn

Fig. 3.

*Measurement of, and Limits of Variation of, the Burned Area.*—A series of unshaved rats were immersed from the base of the skull to the base of the tail, with the lateral and ventral burned area varied by arching the back. Examination of the internal surfaces of the skin of these burned rats showed the burned area to be sharply demarcated from the uninjured area. The skins were smoothed out just enough to allow them to lie flat when pinned to a flat cork surface. The perimeter of the burned area was traced on a piece of cleared x-ray film with a glass marking pencil and the tracing measured by a planimeter (Fig. 3). The relationship of the burned area to the unburned area was expressed in terms of per cent of the total body surface, which was calculated from the formula  $S = KW^{0.60}$  ( $K = 12.54$ ), given by Lee<sup>2</sup> for the white rat.

By varying the curvature of the back on immersion from that in which the belly is covered by water to that in which the water covers only the dorsal surface, the burned area can be varied from 45 per cent to 10 per cent of the total body surface without involving the extremities or the tail. It was found that with practice, using anatomical landmarks, any desired per cent surface within the above range could be reproduced within  $\pm 2$  per cent. The survival data reported below has been obtained for rats receiving burns of 20 per cent, 32 per cent, 36 per cent, and 45 per cent, with a maximum variation of  $\pm 2$ .

Skin measurements on three groups each of four and eight rats sacrificed at twenty-four hours following the burn showed a consistent variation in per cent surface burned of  $\pm 2$ . However, in all experimental groups in which the survival rates were checked for ten days, the burned areas of the surviving rats sacrificed at ten days were found consistently to average about 6 per cent less than those in the same groups dying within seventy-two hours following the burn. Hence, in all experimental groups two checks on the uniformity of the burned surfaces were obtained. All animals within any experimental group having burned areas not falling within the desired range were eliminated.

Percent Survival of Untreated Scald Burned Rats.  
(32% surface burn.)

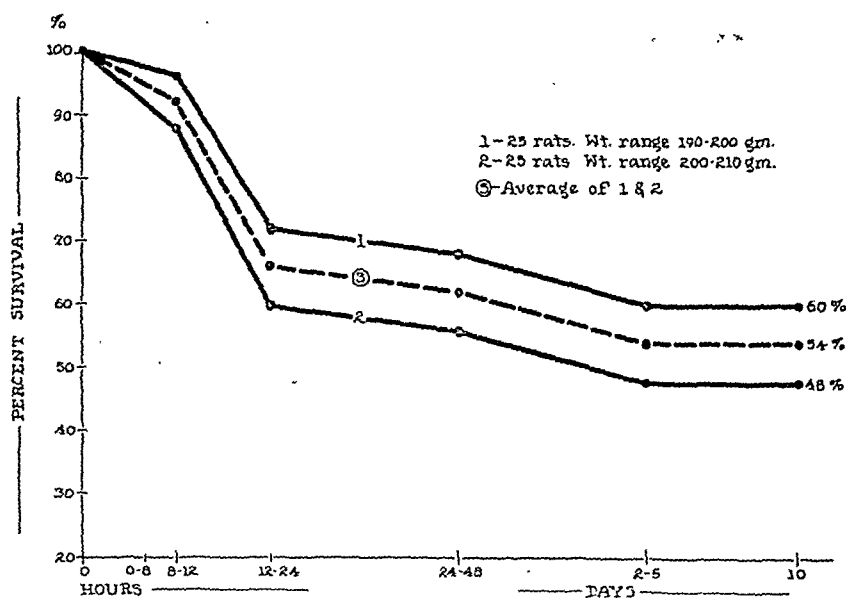


Fig. 4.

*The Relationship of the Burned Surface to Survival.*—Preliminary experiments showed that in order to obtain reproducible survival percentages for any given per cent burn administered according to the conditions outlined, the initial weights of the burned rats must fall within a closely selected weight band. Accordingly, two weight bands have been studied extensively: (1) 190 to 200 grams and (2) 200 to 210 grams. Within these two weight groups, all rats receiving burns of 35 per cent or more of their total body surface have died within forty-eight hours following the burn (ten rats in each weight group), and all rats receiving burns of 20 per cent or less of their total body surface have survived (ten rats in each weight group). Those animals receiv-

ing a burn between 30 and 34 per cent of their total body surface, produced by immersion from the seventh cervical vertebra to the first caudal vertebra and to such a depth that a lateral line would pass through these two points, the Glenovertebral angle of the scapula and the third trochanter of the femur, have shown a consistent average survival percentage for the combined weight groups of 54 per cent. In Fig. 4 is shown the per cent survival of twenty-five rats in each weight group and the average survival of the combined groups. The difference in per cent survival between these two weight groups is not statistically significant; however, because of the variation, equal numbers of animals in the two weight groups were used in all experiments.

*Relationship of Sex to Survival.*—Both male and female rats were tested under the standard conditions. No significant difference in survival was found. Consequently, both male and female rats were used in approximately equal numbers.

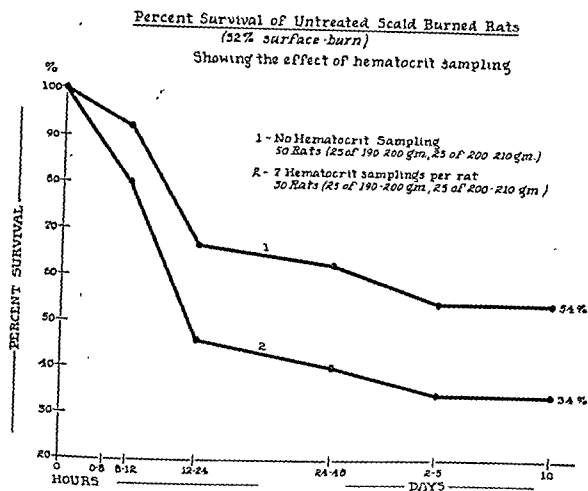


Fig. 5.

*Effect of Manipulation on Survival.*—Early analysis of the data showed that the handling of burned rats and the resultant blood loss, although small, in taking hematocrit samples markedly influenced survival. These samples, seven from each rat, were obtained by loosely rolling the unanesthetized animal in a towel, nicking the lateral tail vein, and drawing up 0.01 c.c. of blood into a uniform bore capillary tube, after which the wound was closed by applying pressure through a sterile gauze sponge and by sealing with a drop of collodion. In Fig 5 is shown a significant decrease in survival between the fifty unhandled animals, curve 1, and the fifty animals subjected to hematocrit sampling represented in curve 2 corresponding to a probability value of between 0.02 and 0.01 as calculated from the Chi square formula of Fisher.<sup>3</sup>

*Effect on Survival Resulting From Various Deviations From the Standard Procedure.*—The survival rates recorded in Figs. 4 and 5 for the 32 per cent burn were reproducible only when all variable factors were rigidly controlled.

Four or more animals were used in each experimental group and in no experimental group included in the data given did all of the animals survive or die when the standard conditions obtained. Hence, small changes in the standard conditions described below which caused all of the four or more of the tested animals either to survive or to die were considered real, although the numbers of tested animals were too small to be treated statistically.

With all other factors constant, a lowering of the bath temperature of  $2^{\circ}\text{C}$ ., that is,  $88^{\circ} \pm 0.25^{\circ}\text{C}$ . for fifteen seconds, permitted 100 per cent survival of ten rats tested.

A reduction of the immersion time from fifteen to ten seconds, other factors constant, allowed a 100 per cent survival of ten rats tested. Increasing the immersion time from fifteen to twenty seconds reduced the survival of ten rats tested to 20 per cent.

Fifteen larger animals weighing between 220 and 230 grams when subjected to the standard burn conditions all survived. However, with other conditions standard, all rats ranging in weight from 220 to 350 grams died when given a 45 per cent  $\pm 2$  body surface burn.

The experiments giving the survival rates recorded in Figs. 4 and 5 were done in the months from November to May, inclusive. In June, however, twenty-one of twenty-two (95.5 per cent) rats tested survived. These experiments were done with a mean elevation in room temperature of  $2^{\circ}\text{C}$ . and an increase in average humidity from 52 to 62 per cent. All other experimental conditions were maintained as outlined in the standard procedure. The survival rate of these twenty-two animals when compared to that of any group of twenty-two of the 100 rats reported in Figs. 4 and 5 shows a statistically significant difference. Other factors than those of temperature and humidity, for example, seasonal variations in quantity and character of the fur and seasonal blood volume changes, may be responsible for the increased survival rate.

#### DISCUSSION

The back burn procedure outlined has several advantages over the complete immersion type of burn for a particular portion of the body, especially when it is desired to maintain the animals over a long period of time postburn for study. The animals are not able to eat the injured tissue, thus avoiding additional trauma and hemorrhage. The extremities remain functional, allowing the animal freedom of movement and allowing the investigator untraumatized veins for infusion and blood-sampling procedures.

It was found that rats burned to the axillas were usually unable to void urine or defecate normally after seventy-two hours following the burn as a result of scab formation on the external genitals and anus. These tissues are not affected as a result of the back burn and hence no complications of this nature arise.

Intraperitoneal complications arising when the ventral body surface was burned were also avoided by the back burn.

Since the burned area can be easily removed upon the death or sacrifice of the animal, accurate measurements of it can be made, thus permitting comparisons of uniformly injured surface areas.

From the data presented it can be concluded that the reproducibility of the recorded survival percentages is critically dependent on the standardization of all conditions incident to the method. Although other strains of rats were not tested, it is possible that the data recorded for this procedure may not be reproducible for other strains of rats.\*

#### SUMMARY

A standard back burn procedure for the white rat (Wistar strain) is described. Survival rates are recorded for the following percentages of the total body surface burned: 20 per cent, 32 per cent, and 45 per cent (all  $\pm 2$ ). Environmental and bath temperature changes, humidity variations, seasonal change, variability in immersion time, animal variables including strain, weight, sex, and condition, and manipulations to which the animals may be subjected are all discussed in relation to their effect on survival.

Because of the sensitivity of the method to changes in any one of several variable factors, it is recommended that simultaneous controls always accompany experimental groups.

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\*Subsequent work done on Wistar rats from the breeding stock since this paper went to press has shown that in order to obtain survival rates similar to those reported, it was necessary to increase immersion time while keeping all other factors constant. Fifty per cent survival was obtained by immersing for twenty seconds. A 100 per cent mortality was obtained by immersing for thirty-five seconds.

## HYDROLYZED CASEIN (CAPAIN) AS A PLASMA SUBSTITUTE

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THE continuing attempts to find a suitable plasma substitute indicate, since the use of Bayliss' gum solution, its need in many cases of surgical shock and allied conditions. In order to find a plasma substitute which combines a considerable water-binding power with complete tolerability, we manufactured a protein hydrolyzate from casein by papain, "capain," showing the following properties:

1. The hydrolyzate is prepared by digesting commercial casein in buffer solution ( $\text{pH} = 5.8$ ) with unactivated papain at  $70^{\circ}\text{C}$ . All unhydrolyzed protein in the resulting mixture is precipitated by adding trichloroacetic acid up to a final concentration of 3 per cent. The trichloroacetic acid is removed by boiling at slightly alkaline reaction, the pH is adjusted, and the solution is concentrated to the desired strength, usually to a nitrogen content corresponding to a 15 per cent protein solution. A suitable preservative may be added. The capain solution is sterilized by boiling once more and kept in sealed glass bottles; it can be stored for over a year (or longer) without cloudiness or other interferences with suitability.

2. Preliminary tests have shown, that although free amino acids may occur, most of the hydrolyzate is in the form of polypeptides, the average degree of hydrolysis having proceeded to the octo-peptide stage. Tests for important amino acids are all strongly positive, especially so when a small amount of lactalbumin had been added to the casein before digestion.

In Fig. 1 is illustrated the water-attracting power of a 13 per cent capain solution compared with that of 2 and 5 per cent sodium chloride solutions, to 10 per cent glucose, and to human plasma. Care has been taken that not only the initial but also the prolonged attraction of water through cellophane was tested. It is seen that the oncotic attraction of the capain solution is about seven times the attraction by plasma; this can also be observed by setting capain and plasma against each other in an osmometer. Capain will exert a considerable osmotic pressure (more than 2 M. of water) against plasma and uphold it for ten hours or more.

The strength of a capain solution may be evaluated by a Kjeldahl nitrogen estimation or by polarimetric or osmometric measurement. All three kinds of determination give proportional results.

The cellophane ultrafiltrate of capain contains about one-third of its nitrogen content, so roughly two-thirds of the nitrogenous material does not pass through cellophane. The depression of the freezing point of capain ultrafiltrate is  $-0.85^{\circ}\text{C}$ ., so it must be considered as slightly hypertonic.

3. We can make the following statements about the pharmacology and immunology of capain injections:

(a) In rabbits, capain was very effective in restoring the blood pressure lowered by extensive bleeding to such degree that the infusions of salt solutions

could not prevent severe hypotension. In normal animals injections of a large amount of capain (2 c.c. per kilogram) caused a slight increase in blood pressure; no other symptoms were observed.

It was not possible to sensitize rabbits by previous injections of capain in the usual way; no anaphylactic or cutaneous reactions developed and no precipitin was formed. We consider, however, that the testing of a solution for the treatment of shock cannot be carried out by animal experiments alone; the human reaction is so much more sensitive that in our opinion the final evaluation can be made only on human subjects (recorded under 3 [b]).

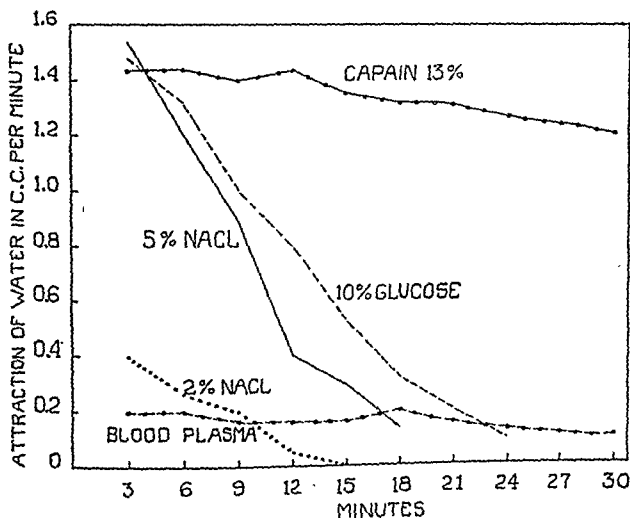


Fig. 1.

(b) Capain was given intravenously in increasing doses up to 20 c.c. to normal persons and to suitable patients; no reactions, with the exception of slight headache and facial heat, were observed; any cutaneous reactive sensitivity to capain was absent. Capain infusion was given in the umbilical veins in prematurely born infants, with a view to provide protein feeding; no food was given orally. It was shown by Dr. J. Jonxis, in the Rotterdam pediatric clinic, that a positive nitrogen balance could be maintained in this way. Also in the newborn infant and in older children the suitability of this hydrolyzate for intravenous protein nutrition was demonstrated. We consider these observations as better proofs for the nontoxicity of capain infusions in human patients.

In the Groningen pediatric clinic a number of starved children (about forty) with "infectious toxic shock" symptoms was successfully treated (in regard to the shock symptoms) by capain infusions. This is another indication that, in the treatment of shock by capain infusions, toxic effects are not to be expected.

The possibility of protein growth from the intravenous injection of a casein digest not completely hydrolyzed may be in accordance with the assumption

tion of Madden and Whipple<sup>1</sup> and others who showed that protein-starved dogs can be kept in nitrogen equilibrium by the intravenous injection of dog plasma without detectable increase in blood amino acid concentration. The hypothesis of realignment of larger polypeptides for intracellular protein synthesis without complete breakdown to amino acids is sustained by the experiments with tagged amino acids and by the observation of Fischer,<sup>2</sup> showing that protein growth in tissue cultures can in many cases be better maintained by a protein hydrolyzate in which the enzymatic breakdown has not progressed too far.

(c) Next to the water-attracting property of capain, a vasoconstrictor activity must be reckoned with. Arteriolar constriction was directly observed and filmed in a Lewis chamber in the rabbit's ear; its onset regularly was ten minutes after the intravenous injection of capain. Moderate venoconstriction not interfering with the infusion was seen in several cases by Dr. Jonxis during injection in an exposed vein in newborn infants.

4. Clinical experiences. The infusion of a solution containing capain for the treatment of various kinds of surgical circulatory shock was studied in the Groningen surgical clinic in seventy cases and was reported by one of us (L. A. (t. H.) in the Communications of this Clinic, Vol. 5, p. 242. The treatment was uniformly successful in regard to the re-establishment of normal minute volume and arterial blood pressure, so that it has now become a routine procedure. In a large number of comparable cases outside the Groningen clinic, which we have not observed, the general result appears to have been the same. A comparison with whole blood could be made in about fifteen patients, and the result is certainly not unfavorable toward the capain infusion. In patients with a very low hemoglobin content whole blood is, of course, indicated.

A certain number of chills was observed (about ten), especially during very rapid injections. They were, as a rule, not severe and in every instance promptly responded to treatment; other deleterious effects were not present. Thrombosis was not observed, although the infusion was sometimes prolonged for from two to three days. In some cases the vasomotor action was clinically evident by a quick response of the blood pressure before the circulatory volume could have increased sufficiently.

The routine procedure in nearly all cases of surgical shock, characterized by low venous pressure, was rapid infusion of saline or glucose solution, to which 100 c.c. of capain per liter were added; in prolonged infusions the capain concentration may be lowered. The increase in both arterial pressure and venous filling must guide the rate of capain infusion; in severe cases it cannot be given too rapidly. The average total amount of capain per patient was from 200 to 300 c.c.; in extreme conditions, a total up to 800 c.c. has been given.

Capain is much simpler to store and administer than is plasma; it keeps indefinitely at room temperature, may be boiled, and is used by simply pouring into the saline infusion. This must be an advantage in field conditions, especially so in the Tropics.

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## STUDIES ON HYPERTENSION

### V. EFFECT OF HIGH SPINAL ANESTHESIA ON THE BLOOD PRESSURE OF PATIENTS WITH HYPERTENSION AND FAR-ADVANCED RENAL DISEASE—ITS POSSIBLE RELATIONSHIP TO THE PATHOGENESIS OF HYPERTENSION

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PREVIOUS investigations<sup>1,2</sup> have led us to doubt that essential hypertension is due to an abnormality of the renal pressor mechanism—renin, renin substrate, and angiotonin. This doubt resulted from studies of the effect of spinal anesthesia on the blood pressure of patients with essential hypertension, the effects of various agents including angiotonin on the blood pressure of these patients at the time of the fall produced by spinal anesthesia, and the bioassay for angiotonin in blood from normal patients, those with hypertension, and others made hypertensive by intravenous injection of angiotonin. In general, the patients previously studied had essentially normal renal function or at least did not have nitrogen retention.

Proponents of the humoral etiology of essential hypertension<sup>3</sup> have attempted to explain the considerable and long-lasting fall in blood pressure which may be caused by various operations on the sympathetic nervous system on the basis of a decrease in vasomotor tonus to the kidney alone. This is postulated to result in improvement of the renal blood flow and a consequent decrease in the production of renin by the kidney. While we have called attention to the unlikelihood of this in previous publications<sup>2,4</sup> based on the studies mentioned, we believe that additional information bearing on this point might result from a comparison of the effects of high spinal anesthesia on the blood pressure of patients with hypertension and normal renal function with the effects similarly obtained on patients with hypertension and far-advanced renal arteriolar disease as shown by the presence of uremia.

Since the present study was undertaken, Corcoran and Page<sup>5</sup> have shown that there is no increase in diodrast and inulin clearances in patients whose blood pressures have been caused to fall by surgery on the sympathetic nervous system. Our studies<sup>7</sup> have shown clearly that there is a decrease in inulin and diodrast clearances in patients whose blood pressure has been caused to fall by spinal anesthesia.

The present study is particularly pertinent in view of Corcoran's<sup>5</sup> statement, "It is only those (patients with hypertension) in whom organic arteriolar disease is absent or minimal who consistently show evidences of vasomotor origin." It must be agreed that patients who have uremia resulting from essential hypertension have "far-advanced arteriolar disease" at least in the kidneys.

It is for these reasons that these studies have been made on patients with uremia. Our original approach in this direction was dictated by our belief that the elevated blood pressure of hypertensive patients with far-advanced renal arteriolar disease would not fall as much under spinal anesthesia as the

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TABLE I. EFFECT OF SPINAL ANESTHESIA WITH 150

PATIENT	SEX	AGE (YR.)	N.P.N. (MG. %)	UREA NITROGEN (MG. %)	MAXIMUM CONCENTRATION TEST	BLOOD PRESSURE BEFORE SPINAL ANESTHESIA	BLOOD PRESSURE DURING SPINAL ANESTHESIA†	DURATION OF SIGNIFICANT BLOOD PRESSURE FALL (MIN.)	LEVEL OF SENSORY ANESTHESIA	MOTOR PARALYSIS LOWER LIMBS	MALIGNANT PHASE	URINARY FINDINGS
S. B.	M	38	75	63	1.018	243/190	120/90	60	Slightly above nipple line	Yes	Yes	Albumin, 3+; gross blood; many casts
L. S.	F	44	120	75	1.018	226/108	124/70	76	2nd I.S.	Yes	Yes	Albumin, 2+; 4 to 5 R.B.C. per H.P.F. occasional cast
R. M.	F	46	81	51	1.012	240/142	126/80	37	2nd I.S.	Yes	No	Albumin, 4+; many pus cells and casts
C. S.	M	44	171	116		244/160	162/138	42	2nd I.S.	Yes	No	Albumin, 4+; many R.B.C. and pus cells; occasional cast
E. F.	F	49	123	90	1.009	242/124	75/50	86	Unable to cooperate	Yes	Yes	Albumin, 3+; frequent R.B.C., W.B.C., and casts
L. L.	M	54	235		1.026	218/108	186/90	53	2nd I.S.	Yes	No	Albumin, 3+; gross blood; 3 to 5 W.B.C. per H.P.F.; occasional cast
A. B.	F	21	81		1.012	250/164	220/146	49	3rd I.S.	Yes	No	Albumin, 3+; numerous pus cells and casts
E. H.	F	40	200	75	1.017	178/100	160/72	63	3rd I.S.	Yes	No	Albumin, 4+; many W.B.C.; occasional R.B.C.
T. D.	F	52	120	64	1.007	234/124	210/98	57	Clavicle	Yes	No	Albumin, 4+; frequent casts, R.B.C., and W.B.C.
W. C.	M	65	85	60	1.015	226/134	186/102	34	Xiphoid	Yes	No	Albumin, 4+; 8 to 10 R.B.C. per H.P.F.
H. R.*	M	44	300	202	1.009	202/128	186/108	43	Xiphoid	Yes	No	Albumin, 2+; many R.B.C., W.B.C., and casts
H. R.*	M	44	300	202	1.009	228/138	184/112	72	2nd I.S.	Yes	No	Albumin, 2+; many R.B.C., W.B.C., and casts
T. R.	M	34	200	140	Not done	178/100	156/100	80	Slightly above xiphoid	Yes	No	Anuria; albumin, 4+; many R.B.C., W.B.C., and casts

\*Same patient.

†Lowest level of fall.

No. of PROCAINE HYDROCHLORIDE ON BLOOD PRESSURE

OCULAR FUNDI	ELECTROCARDIOGRAM	NECROPSY FINDINGS	REMARKS
Usual hypertensive changes; many hemorrhages and exudates	T <sub>1</sub> , diphasic; T <sub>2</sub> and T <sub>3</sub> , neg.	Not done	R.B.C., 3.2 M; Hb., 60%
Papilledema, hemorrhages, and exudates	S-T <sub>1</sub> and S-T <sub>2</sub> , depressed; T <sub>1</sub> and T <sub>2</sub> , neg.	Arteriolar nephrosclerosis	R.B.C., 3.5 M; Hb., 66%
Papilledema, hemorrhages, and exudates	Slurred QRS; T <sub>1</sub> , T <sub>2</sub> , and T <sub>3</sub> , neg.; S-T <sub>1</sub> and S-T <sub>2</sub> , depressed	Did not die while observed	R.B.C., 3.2 M; Hb., 62%
Usual hypertensive changes plus old exudate and hemorrhage	QRS slurred; T <sub>1</sub> , low; T <sub>2</sub> , neg.	Chronic pyelonephritis, bilateral and arteriolar nephrosclerosis	R.B.C., 2.9 M; Hb., 60%
Papilledema, hemorrhages, and exudates	S-T <sub>1</sub> and S-T <sub>2</sub> , depressed; T <sub>1</sub> , T <sub>2</sub> , and T <sub>3</sub> , diphasic	Not done	R.B.C., 2.8 M; Hb., 58%
Hemorrhages, exudates, and papilledema	Myocardial damage	Chronic glomerulonephritis with acute exacerbation	R.B.C., 2.5 M; Hb., 53%; hyperglobulinemia: 6.6 Gm. % globulin; 3.2 Gm. % albumin
Usual hypertensive changes plus hemorrhages and exudates	No myocardial damage	Arteriolar nephrosclerosis plus pyelonephritis	Died at home; kidney removed; R.B.C., 3.0 M; Hb., 58%
Advanced vascular changes	T <sub>1</sub> , T <sub>2</sub> , and T <sub>3</sub> , diphasic; T <sub>4</sub> , neg.	None	Old record indicates evidence of pyelonephritis; pyuria; back pain; R.B.C., 1.54 M; Hb., 38%
Usual hypertensive changes plus hemorrhages and exudates	S-T <sub>1</sub> and S-T <sub>2</sub> , depressed; T <sub>1</sub> , neg.	Arteriolar nephrosclerosis	R.B.C., 2.7 M; Hb., 50%
Old hemorrhages and usual hypertensive changes and exudates	QRS slurred; T <sub>1</sub> and T <sub>2</sub> , neg.	Arteriolar nephrosclerosis	No anemia
Usual hypertensive changes, hemorrhages, and exudates	Not done	Chronic glomerulonephritis	R.B.C., 1.5 M; Hb., 25%
Usual hypertensive changes, hemorrhages and exudates	Not done	Chronic glomerulonephritis	R.B.C., 1.5 M; Hb., 25%
No report	Not done	Patient recovered	Diagnosis: Acute glomerulonephritis; R.B.C., 3.8 M; Hb., 77%

blood pressure of patients with hypertension and normal renal function under the same conditions if the initial blood pressure elevation is due to a humoral substance produced in the kidney. It is logical to assume that interruption of vasomotor impulses to the kidney is not likely to influence favorably the circulation through the kidney which has such extensive and intensive vascular pathology as to result in uremia; and it is pertinent again, in this relationship, to refer to work<sup>7</sup> which has demonstrated a decrease in inulin and diodrast clearances in hypertensive patients whose blood pressure has been caused to fall to normal by interrupting vasomotor function with spinal anesthesia.

#### METHODS

Hypertensive patients with uremia were selected at random. Preliminary control blood pressures and experimental observations of the effect of spinal anesthesia on the blood pressure levels were done as previously described.<sup>1</sup> All of the patients showed clinical evidence of uremia. Renal failure was confirmed by significant elevation of blood nitrogen.

#### RESULTS

In Table I are contained the data obtained in thirteen studies on twelve patients. Ten of them were diagnosed clinically as having essential hypertension, one was diagnosed as having acute glomerulonephritis (T. R.), and one, chronic glomerulonephritis (H. R.). The diagnosis of chronic glomerulonephritis was confirmed by necropsy. In addition, five other patients were studied at necropsy. Necropsy showed that one of those (L. L.) with a clinical diagnosis of essential hypertension had chronic glomerulonephritis with an acute exacerbation. One patient (C. S.), diagnosed clinically as having essential hypertension, was found to have a superimposed chronic bilateral pyelonephritis at autopsy in addition to arteriolar nephrosclerosis. The kidney of one patient (A. B.) was studied after nephrectomy done as result of a mistaken clinical diagnosis of unilateral pyelonephritis as the cause of the hypertension. Pathologic examination of this kidney showed a slight amount of pyelonephritis and primarily arteriolar nephrosclerosis. The nephrectomy had no favorable influence on the hypertension. The patient's clinical course was rapidly and steadily downhill and she died in uremia six months after nephrectomy. The studies summarized in Table I on A. B. were done following nephrectomy and after supervention of uremia. Autopsies on L. S., T. D., and W. C. showed arteriolar nephrosclerosis.

Further analysis of the tabulated data shows that high spinal anesthesia produced a marked fall in both systolic and diastolic blood pressure in five patients (S. B., L. S., R. M., C. S., and E. F.). Of the remaining seven patients whose blood pressure fell only moderately (L. L., A. B., E. H., T. D., W. C., H. R., and T. R.) under the influence of high spinal anesthesia, one (T. R.) had acute glomerulonephritis with anuria, two (L. L. and H. R.) had chronic glomerulonephritis (at autopsy), one (A. B.) had proved pyelonephritis in a kidney removed during life, one (E. H.) had an observed clinical course of from four to five years of pyelonephritis, and the remaining two (T. D. and W. C.) had arteriolar nephrosclerosis proved at autopsy.

The initial level from which the blood pressure falls due to high spinal anesthesia is not a determining factor in the extent of the fall, as can be seen by the fact that blood pressure well over 200 mm. Hg was encountered in both individuals with marked falls as well as in those who showed only moderate drops in blood pressure.

Necropsies were done on two (L. S. and C. S.) of the five patients whose blood pressure showed great falls during high spinal anesthesia. The kidneys showed arteriolar nephrosclerosis in both.

#### DISCUSSION OF RESULTS

Five of twelve patients (S. B., L. S., R. M., C. S., and E. F.) showed extreme falls of blood pressure during high spinal anesthesia. At necropsy two of these (C. S. and L. S.) showed arteriolar nephrosclerosis to be the cause of renal failure. The other three (S. B., R. M., and E. F.) had a clinical diagnosis of essential hypertension with renal failure. Two of the latter three patients (S. B. and E. F.) had clinical manifestations of malignant hypertension. Unfortunately, necropsies were not done on these individuals. These results are in conformity with our original thesis that the blood pressure of patients with sufficiently advanced arteriolar disease to produce uremia should still fall markedly during high spinal anesthesia if the hypertension is of vasomotor origin, and should not fall markedly if the hypertension is of renal humoral origin, any fall being dependent upon improvement in renal blood flow.

Unfortunately, the matter is not as simple as this explanation. This is shown by the fact that seven (L. L., A. B., E. H., T. D., W. C., H. R., and T. R.) of twelve patients failed to show as marked a fall as we obtained in the other five patients and as marked as we have shown in numerous other patients, the study of whom formed the basis of other publications.<sup>1, 2</sup>

In the attempt to explain our failure to obtain marked falls of blood pressure during spinal anesthesia, we analyzed our data further. Three of these seven (L. L., H. R., and T. R.) had acute or chronic glomerulonephritis. The diagnosis of chronic glomerulonephritis was proved by necropsy in two of these (L. L. and H. R.). An additional patient (E. H.) had a justifiable clinical diagnosis of chronic pyelonephritis as shown by our own clinic and hospital records of repeated pyuria, etc., for approximately five years' duration.

We do not understand the significance of the variability in the results obtained in our patients with known nephritis and hypertension. While we realize that it is speculative, it is at least reasonable, on the basis of great accumulation of evidence, to assume that if there is such a thing as hypertension of humoral origin in the human being, it is probably to be found in the individuals with intrinsic renal pathology.<sup>3</sup>

While we have no proof, we assume that the failure to respond with marked drop in blood pressure is explained definitely in three cases (L. L., H. R., and T. R.) and possibly in another case (E. H.) by our further assumption that the pathogenesis of elevated blood pressure is different in essential hypertension and acute and chronic inflammatory disease of the kidney. Unfortunately for this assumption, four patients with proved cases of acute or chronic inflammatory diseases of the kidney (L. L., H. R., T. R., and E. H.) and three with proved cases of arteriolar nephrosclerosis (A. B., T. D., and W. C.) reacted similarly to spinal anesthesia with only moderate drops of blood pressure. In four patients with renal inflammatory disease there was an average fall of 26 mm. Hg systolic and 18 mm. Hg diastolic pressure. In three patients with nephrosclerosis, the average fall was 31 mm. Hg in systolic pressure and 25 mm. Hg in diastolic pressure. While there is a difference, the amount of difference and the few cases do not warrant definite conclusions.

We have not explained failure to obtain a marked fall in blood pressure during spinal anesthesia in three other patients (A. B., T. D., and W. C.), all

of whom had a clinical diagnosis of essential hypertension, two of whom showed at autopsy only arteriolar nephrosclerosis as the cause of renal failure, and one (A. B.) of whom showed, in a surgically removed kidney, arteriolar nephrosclerosis to be the essential lesion plus a slight pyelonephritis.

In the attempt to throw further light on this matter, we have analyzed the data in terms of initial blood pressures. An explanation cannot be found here, as can be seen by the fact that five patients (S. B., L. S., R. M., C. S., and E. F.) had initial systolic blood pressures ranging from 226 to 244. The blood pressures of all these fell markedly during spinal anesthesia. Whereas those patients who did not show much fall during spinal anesthesia (L. L., A. B., E. H., T. D., W. C., H. R., and T. R.) had initial systolic blood pressures of from 178 to 250, five of these (L. L., A. B., T. D., W. C., and H. D.) had initial pressures in the same range as the five who showed such a marked drop.

In view of the acceptance of the idea<sup>10</sup> that renal failure of extreme degree results also in a loss of the metabolic and detoxifying function of the kidney with possible retention of pressor substances, our data have been analyzed from the viewpoint of the difference in the amount of nitrogen retention in patients who showed marked blood pressure falls compared to those who showed slight to moderate falls in blood pressure.

In the five patients who showed extreme falls in blood pressure, the non-protein nitrogen varied from 75 to 171 mg. per cent, with an average of 114 mg. per cent. In the patients (including those with nephritis—W. C., H. R., and T. R.) who showed only slight to moderate falls in blood pressure, the individual variations in nonprotein nitrogen were from 81 to 300 mg. per cent, with an average of 174 mg. per cent.

At present we are unable to draw any definite or far-sweeping conclusions from the fact that the patients in the group showing the greatest falls in blood pressure had less chemical evidence of uremia than did those in the group which showed only slight to moderate falls in blood pressure. We are, however, postulating the possibility that the blood of patients in uremia contains significant and sufficient amounts of pressor substances, such as aromatic amines or phenols, to explain perhaps the variability of the degree of blood pressure fall during spinal anesthesia. Individual susceptibility to drug action must not be disregarded as a further possibility.

#### SUMMARY AND CONCLUSIONS

Falls in blood pressure obtained during spinal anesthesia in patients with essential hypertension and uremia may be very marked and as great as falls of blood pressure similarly produced in patients with essential hypertension and normal renal function.

We believe that the great fall in blood pressure induced by high spinal anesthesia indicates that the elevation of blood pressure is due to increased vasomotor tonus even in patients with extreme irreversible arteriolar disease as shown by clinical and chemical evidence of uremia.

These results are contradictory to the thesis that the fall of blood pressure in hypertension produced by functional or anatomical interruption of the sympathetic nervous system is due to improvement in renal blood flow.

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## THE EFFECT OF INTRAVENTOUS TYPHOID VACCINE ON THE INTRAVENTOUS GLUCOSE TOLERANCE TEST

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THE administration of glucose intravenously to replace that usually given orally in carrying out glucose tolerance examinations has been proposed by McKean and associates.<sup>1</sup> The obvious advantages of the test are reduction in the time required (from several hours to fifteen minutes) and obviation of marked discrepancies on repeated examinations caused by irregularities in rate of absorption. The need of a sufficient number of control tests on normal subjects was the immediate incentive for this investigation which was performed on a group of normal soldiers. Later the tests were repeated on a group of soldiers being treated for gonorrheal infections by fever therapy induced by intravenous injections of triple typhoid vaccine. The inclusion of the initial tests from this latter group with those of the normal subjects appeared warranted on the ground that such a local infection would not significantly alter the results.

The tests were made under basal conditions by the intravenous injection of 50 per cent glucose in physiologic salt solution. The dose was equivalent to 0.2 Gm. per kilogram of body weight. The entire volume of sugar solution was injected in exactly one and one-half minutes and glucose determinations were made by the Folin and Wu method on samples of blood taken precisely at three, four, five, ten, and fifteen minutes after the injections were started, without stasis.

TABLE I. INTRAVENOUS TOLERANCE TESTS

CASE	AGE (YR.)	WEIGHT (POUNDS)	DOSE (CC.)	FAST- ING	BLOOD SUGAR (MG. PER CENT)					DATE	DIAGNOSIS	TEMPERATURE (° F.)	PULSE	RESPIRATION
					POSTINJECTION									
					3 MIN.	4 MIN.	5 MIN.	10 MIN.	15 MIN.					
1	24	138	12.5	74	154	190	157	153	148	12/17/43	G. C.*	98.6	92	18
2	21	140	12.5	71	125	138	158	118	109	12/14/43	G. C.	99.5	110	23
3	22	173	15.7	72	180	184	156	121	100	12/13/43	G. C.	98.6	82	20
4	22	173	15.7	71	178	173	167	101	94	12/18/43	G. C.	98.0	64	18
5	23	165	15.0	70	160		153	109	102	12/13/43	G. C.	97.6	80	18
6	20	135	12.2	66			160	112	103	12/15/43	G. C.	99.0	88	20
7	37	148	13.2	71	153	163	161	153	141	12/17/43	G. C.	99.0	98	20
8	24	172	15.6	70	178	183	174	151	133	12/16/43	G. C.	98.6	90	18
9	37	171	15.5	76	160	167	174	169	138	12/20/43	G. C.	98.0	80	18
10	23	150	13.6	71	168	160	152	131	116	12/20/43	G. C.	98.2	80	18
11	25	140	12.7	68	179	179	169	142	120	12/15/43	G. C.	99.2	84	20
12	22	165	15.0	70	182	157	150	121	87	12/18/43	G. C.	98.6	86	18
13	21	168	15.0	80	182		178	138	107	12/16/43	G. C.	98.6	74	18
14	19	154	14.0	88	187	196	179	144	129	2/ 7/44	Arthritis			
15	26	160	14.5	87	206	228	235	125	107	8/27/43	Normal			
16	28	180	16.8	87	222.2	220	195.2	190.4	173.0	8/12/43	Normal			
17	47	150	12.6	95	202	208	216	164	154	8/19/43	Normal			
18	26	160	14.5	69	160.0	163.0	153.8	153.8	118	8/12/43	Normal			
19	21	160	14.4	87	129.0	160.0	190.4	133.3	125.0	8/11/43	Normal			
20	27	165	15.0	95	153.8	155.0	166.7	142.8	133	3/10/43	Normal			
21	20	160	14.5	108	142.8	144	144.4	153.8	133	8/13/43	Normal			
22	26	140	12.2	91	181.8	167	160.0	153.0	133	8/10/43	Normal			
23	23	120	11.0	98	154	143	137	148	133	8/ 7/43	Arthritis			
24	22	152	13.5	94	175	192	156	133	128	8/ 7/43	Rheumatic fever			

\*Acute gonorrhea.

The results of the tests on the normal control subjects and the preliminary findings in the experimental subjects are given in Table I.

The mean for fasting blood sugar level was  $80.4 \pm 2.4$  mg. per 100 c.c. with a standard deviation of  $11.7 \pm 1.7$ . The mean level at the highest points of the curves, which usually occurred at the fourth minute, was  $174.6 \pm 5.0$  mg. per 100 c.c., with a standard deviation of  $24.6 \pm 3.6$ . The mean value at the end of 15 minutes was  $123.5 \pm 4.1$  mg., with a standard deviation of  $19.9 \pm 2.9$ .

The patients who received the intravenous injections of typhoid vaccine were chosen because the gonorrheal infections had proved resistant to sulfathiazole therapy. The vaccine was the regular triple typhoid used by the Army for inoculation and contained 1,000 million typhoid bacteria and 250 million each of paratyphoid A and B. The amount used for a single injection varied from 25 million to 7,000 million and the number of injections varied from fifteen to twenty for each individual. In each case the amount of vaccine was started at a low level and the concentration gradually increased as it was found that the lower dosages became ineffective. The temperature was raised to from 104 to 106° F. and maintained for several hours. The second glucose tolerance test was taken after the fever had reached the desired temperature. The effect of the typhoid vaccine is shown in Table II. There was a significant increase in temperature from 98.4 to 105.1° F., in pulse rate from 80.0 to 120.2 per minute, and in respiratory rate from 18.6 to 29.5 per minute.

There was no significant difference between the mean values for initial blood sugar level of 71.7 mg. per 100 c.c. when the temperature was normal and that of 78.9 mg. per 100 c.c. when the temperature was elevated. The mean values for the three-minute interval when the blood sugar reached its highest



TABLE II. INTRAVENOUS GLUCOSE TESTS DURING FEVER

CASE	TOTAL MILLION TYPHOID BACTERIA	NUMBER OF DOSES	AMOUNT EACH DOSE	TIME FOR DOSES (HR.)	DURATION FEVER AT TEST (HR.)	BLOOD SUGAR (MG. PER CENT)					TEMPERATURE	PULSE	RESPIRATION	
						FAST- ING	POSTINJECTION							
							3 MIN.	4 MIN.	5 MIN.	10 MIN.				15 MIN.
3	825	17	25-100	11	1	74	174	155	147	98	96	104.9	120 24	
4*	29,200	10	200-5000	7½	5	91	193	198	182	157	154	103.0	130 25	
5	1,300	17	50-200	9	1	76	159	146	138	125	115	104.8	120 35	
6	6,500	20	100-400	9½	3½	70	174	167	160	131	118	104.0	120 26	
9	15,100	19	50-2000	7	12½	93	177	167	170	165	148	105.4	120 35	
10*	15,100	19	50-2000	7	12½	72	143	136	131	119	105	105.4	120 25	
11	4,700	18	100-400	8	13	71	163	167	157	138	133	105.0	115 30	
12	1,300	16	50-200	9	3	85	154	156	160	154	142	104.8	108 28	
12	28,200	15	200-4000	7½	4½	70	169	151	134	126	120	105.0	114 32	
13	18,700	19	100-7000	9	4	87	195				145	106.5	135 35	

\*Developed, school.

\*Developed shock.

value were 173.2 mg. for the control test and 170.1 mg. during the fever. The difference between these values was not significant

At the fifteen-minute interval the mean blood sugar level was 105.4 mg. for the control test and 127.6 mg. for the test taken during the period of hyperpyrexia. The difference between these readings was 22.2 mg. In eight of the ten subjects, the level taken during the fever was higher than that taken in the control period; in one instance the levels were essentially the same and in the remaining subject the control level was significantly higher. In view of this fact, this difference may be considered significant. The findings are consistent with those of Looney and Borkovic<sup>2</sup> in which the hyperpyrexia was produced by diathermy.

It would appear, therefore, that as a result of fever, no matter what its origin, there is a slowing down in the removal of sugar from the blood. In this regard, one is reminded of the acidosis which frequently occurs in infants and young children during slight fevers and it suggests the possibility that this may be due to a greater susceptibility of the sugar metabolism in these individuals' disarrangement by fever.

The enormous quantities of triple typhoid vaccine which may be administered intravenously is strikingly demonstrated in this series. In half of the subjects, doses of from 2 to 7 c.c. of undiluted vaccine were administered. That the treatment is not without danger was seen by the fact that two of the patients (see Table II) developed shock which in one (Case 10) was so severe as to cause considerable anxiety to the investigators. The shock occurred in these subjects despite the fact that they had on several previous occasions received doses of vaccine as great as they were being given at the time. As a result of this development and because there appeared to be no way of predicting when such a collapse might occur, the investigation was discontinued.

## SUMMARY

Glucose tolerance curves were obtained after the intravenous administration of 0.2 Gm. of 50 per cent glucose per kilogram of body weight in twenty-four essentially normal soldiers. The blood sugar level rose from a mean of 80.4 mg. per 100 c.c. to a maximum of 174.6 mg. about the fourth minute and then dropped to 123.5 mg. at the fifteenth minute.

The glucose tolerance curve was elevated after the intravenous administration of triple typhoid vaccine for the treatment of sulfathiazole-resistant

gonorrhea. The mean level at the end of fifteen minutes was 22.2 mg. higher than the initial level of 105.4 mg.

The effect is probably due to the induced fever and not to any specific effect of the vaccine.

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## THE DIAGNOSIS OF ENTEROBIASIS

### COMPARATIVE STUDY OF THE GRAHAM AND HALL TECHNIQUES IN THE DIAGNOSIS OF ENTEROBIASIS

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WE HAVE previously<sup>1</sup> carried out a comparative study of the techniques of Hall<sup>2</sup> (N.I.H. swab) and of Graham<sup>3</sup> (Scotch cellulose tape loop) for the diagnosis of enteriobiasis. We used both methods, on five successive days, in the examination of seventeen children heavily infected with *Enterobius vermicularis*. Positive results were obtained in 96.5 per cent with Graham's method and in 91.7 per cent with Hall's. In view of the fact that the children in the group tested were heavily parasitized, the comparison was repeated in 240 school children who had not been previously examined. In this second series, 45 per cent positive findings were obtained with Graham's method and 30 per cent with Hall's. Based on these results, we reached the conclusion that the Graham method is the technique to be recommended in the younger age groups, since in adults we considered the Hall technique to be easier to use.

We have subsequently continued to use the Graham method both in children and in adults and have confirmed its superiority by the studies mentioned. It was therefore considered advisable to present the results of these additional studies in 430 individuals, mostly adults; all of whom were examined by both of the techniques under investigation.

#### MATERIALS AND METHODS

The Graham method was used with the slight modification suggested by us, which consists of using a strip of cellulose adhesive tape 5 cm. in length, without folding the ends, grasping the loop lengthwise with a dissecting forceps. This modification reduces the amount of time required to prepare the loop and the amount of material necessary. The Hall method was carried out exactly as described by Hall.

The examinations were made on the patients of a Surgical Hospital in Mexico City. Each individual was first examined by the Hall technique and then additional material was collected by means of the Graham method.

TABLE I. RESULTS OBTAINED IN EXAMINATION OF 430 INDIVIDUALS WITH THE TECHNIQUES OF HALL AND GRAHAM

AGE GROUPS (YE.)	MALES										FEMALES										TOTAL MALES AND FEMALES									
	HALL					GRAHAM					HALL					GRAHAM					HALL					GRAHAM				
	TOTAL NUM. DEE	NUM. BER TIVE	PER CENT POST-	PER CENT FISH-	TOTAL NUM. BER	TOTAL BER TIVE	PER CENT POST-	PER CENT FISH-	TOTAL NUM. BER	TOTAL BER TIVE	PER CENT POST-	PER CENT FISH-	TOTAL NUM. BER	TOTAL BER TIVE	PER CENT POST-	PER CENT FISH-	TOTAL NUM. BER	TOTAL BER TIVE	PER CENT POST-	PER CENT FISH-	TOTAL NUM. BER	TOTAL BER TIVE	PER CENT POST-	PER CENT FISH-						
11-20	37	10	27	37	19	51	10	24	42	18	43	20	70	37	25	70	37	25	70	37	25	70	37	25	70					
21-40	75	10	13	75	19	25	18	15	118	34	29	38	193	53	14	193	53	14	193	53	14	193	53	14	193					
41-60	56	8	14	56	16	29	69	8	62	69	26	16	135	34	13	135	34	13	135	34	13	135	34	13	135					
61-80	17	3	18	17	6	35	16	1	16	6	38	4	33	12	12	33	12	12	33	12	12	33	12	12	33					
Totals	185	31	17	185	60	32	245	37	15	245	76	31	430	68	16	430	136	32	430	136	32	430	136	32	430					

## RESULTS

As can be seen from Table I, the Graham method proved to be much more efficient than the Hall technique. The total percentage of positive findings was 32 per cent with the Graham method and 16 per cent with the Hall method. In all age groups, without regard to sex, the percentage of positive results obtained with the Graham method was significantly higher.

## DISCUSSION

The findings presented confirm the superiority of the Graham method, even in adults with perianal hairs, which includes almost all the adult males. The considerable percentage of positive findings obtained justifies the use of this procedure also in adults, since the minimal discomfort which may be occasioned by its use is well tolerated by all patients.

As indicated previously, in each case, material for the Hall method was collected first so that actually it enjoyed a small advantage over the Graham method.

## SUMMARY

Examinations for the diagnosis of enterobiasis were carried out in 430 individuals using both the Hall and Graham techniques. The percentage of positive results obtained was 32 per cent with the Graham method and 16 per cent with the technique of Hall.

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## THE URINARY EXCRETION OF ANTIPERNICIOUS ANEMIA FACTOR

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A NUMBER of investigators have suggested that the antipernicious anemia factor (A.P.A.) may be excreted in the urine of human subjects;<sup>1, 2, 13</sup> the evidence offered in support of this possibility, however, has not been conclusive. Since the A.P.A. factor\* may be a relatively small molecule,<sup>3</sup> one would not be surprised to find that it passed readily through the glomerular filter, so that after the injection of a large amount of liver extract, a portion of the active principle might be found in the urine. However, the administration of 300 U.S.P. units of liver extract in one intramuscular injection to a patient with pernicious anemia in relapse has been observed to induce a remission which lasted for nine months,<sup>4</sup> and Seymour, Heinle, and Miller<sup>5</sup> found that when

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\*That the liver principle may be multiple in nature, as claimed by Jacobson and Subbarow,<sup>3</sup> is realized. For the sake of convenience, however, it will be referred to as A.P.A. factor rather than factors.

patients in remission were given from 60 to 140 U.S.P. units of liver extract over a short period of time, relapse did not occur for from nine to thirty weeks. These observations suggest that the urinary loss of the injected active principle could not have been great, but they must be interpreted with caution because Goldhamer<sup>6</sup> has shown that the deficiency of intrinsic factor in gastric secretion is relative rather than absolute. The amount of supplementary A.P.A. factor required daily by patients with pernicious anemia, therefore, probably varies with the degree of deficiency of the intrinsic factor. This study was undertaken to determine more definitely how much of the A.P.A. factor is excreted promptly into urine after parenteral administration of liver extract.

In previous studies it has been shown<sup>7-12</sup> that a substance is present in the urine of normal individuals and of patients with pernicious anemia which is capable of stimulating the production of reticulocytes in pigeons, rats, and guinea pigs. However, since this reticulocytogenic substance is not known to be identical with, or related to, the A.P.A. factor, investigations using these test animals have not provided conclusive evidence that the A.P.A. factor is found in human urine.

A more satisfactory approach to the problem has been made by investigators who have used patients with pernicious anemia in relapse for assaying urine extracts. Decastello<sup>2</sup> reported in 1935 that urine from normal subjects and from patients with pernicious anemia, when given rectally or intramuscularly, was effective in the treatment of pernicious anemia. However, his results were not uniform, and Jequier and Apsey<sup>13</sup> were not able to confirm them. Wakerlin,<sup>14</sup> after reporting that a patient with pernicious anemia did respond to intramuscular injection of a urine extract, was unable to duplicate his results.<sup>15</sup> Thornley<sup>16</sup> was not able to obtain A.P.A. activity from 1,750 c.c. of normal human urine with a method of extraction which had consistently yielded potent anti-anemia fractions from liver; his assay was done on a patient with pernicious anemia in relapse. In 1940, Wilkinson<sup>1</sup> found that extracts made from 20 to 40 liters of urine obtained either from normal individuals or from patients with pernicious anemia in remission induced hematologic responses in several patients with pernicious anemia in relapse; similar extracts prepared from the urine of untreated patients with pernicious anemia had no therapeutic value. He used two methods of extraction: (1) The urine was concentrated directly, taken up in 90 per cent alcohol, reconcentrated, precipitated in absolute alcohol, and dried; (2) the urine was treated with Norite, eluted from the Norite by 60 per cent alcohol, concentrated, precipitated in absolute alcohol, and dried.

Because Wilkinson's observations provide the strongest evidence so far accumulated that the principle in liver effective in the treatment of pernicious anemia is excreted in urine, his data are reviewed in more detail as follows: (1) Extracts made from the urine of normal individuals were injected intramuscularly in doses equivalent to from 8.5 to 20 liters into three patients with pernicious anemia in relapse. Reticulocyte responses of 14.8, 11.6, and 12.5, respectively, were stimulated, and a rise in the red cell count of 0.77 and 0.62 million per cubic millimeter occurred in two patients; erythrocyte values were not reported for the third patient. (2) Extracts made from the urine of patients with pernicious anemia in remission and given intramuscularly in doses equivalent to from 10 to 22.6 liters to three patients with pernicious anemia in relapse caused reticulocyte responses of 12.2, 29.6, and 15.2 per cent and rises in the erythrocyte level of 0.82, 1.34, and 1.44 millions per cubic millimeter.

(3) Extracts made from the urine of patients with pernicious anemia in relapse failed to induce a reticulocyte response. It is emphasized that Wilkinson used large amounts of urine for his extracts and made no attempt to determine whether the A.P.A. factor from injected liver extracts was excreted in significant amounts.

In the study which forms the basis for this report, it was determined that urine excreted by subjects during the twenty-four hours following a relatively large injection of liver extract contained no detectable A.P.A. activity. Control observations demonstrated that the method used for extracting urine was satisfactory; A.P.A. factor which had been added to urine directly could be recovered.

#### METHODS

The simplest possible method of extraction was used in order that loss of activity in the extraction process might be minimized. Urine was evaporated to near dryness under reduced pressure at 46° C. Then 95 c.c. of 70 per cent ethyl alcohol were added and the mixture filtered; the filtrate was evaporated nearly to dryness under reduced pressure at a temperature not greater than 50° C. The residue was taken up in sterile water to give a final volume of from 27 to 40 c.c., and phenol was added as preservative to give a final concentration of 0.5 per cent.\* The solid content of each preparation was determined. Each extract was cultured aerobically and anaerobically and used only after it had been found to be sterile. Subcutaneous injection of 1 c.c. into a dog produced only slight induration after twenty-four hours. All of the extracts caused pain at the site of intramuscular injection. In order to lessen the discomfort, the daily dose was diluted with water (from 2 to 8 c.c.). One cubic centimeter of 1 per cent novocain was added to the syringes for administration of Extracts 2 and 4.

Four extracts were prepared:

*Extract 1.*—Ten cubic centimeters of a commercial liver extract† containing 100 U.S.P. units of A.P.A. factor were added to 1 liter of normal human urine before it was evaporated. Solid content of the extract equalled 36.5 per cent and final volume was 27 c.c.

*Extract 2.*—Ten cubic centimeters of a commercial liver extract† containing 100 U.S.P. units of A.P.A. factor were added to 1 liter of normal human urine before it was evaporated. Solid content of the extract equalled 55.9 per cent and final volume was 30 c.c.

*Extract 3.*—A healthy adult male (R. H. R.) was injected intramuscularly on each of three successive days with 10 c.c. of a liver extract† containing 100 U.S.P. units of A.P.A. factor. Urine was collected for a twenty-four hour period beginning with the last injection. This urine (1,175 c.c.) was extracted. Solid content was 59 per cent and final volume was 40 c.c.

*Extract 4.*—A patient with pernicious anemia in relapse (E. G.), who had been given a daily injection of 1 U.S.P. unit of A.P.A. factor for fifteen days, was on each of five subsequent days given intramuscularly 5 c.c. of Reticulogen† containing approximately 100 U.S.P. units per 5 c.c. On the fifth day, a twenty-four hour clean-voided urine specimen (1,790 c.c.) was collected and extracted. Solid content equalled 51.8 per cent and final volume was 30 c.c.

Red blood cell counts, hemoglobin levels, and reticulocyte percentages were determined daily on the capillary blood of the test subjects. Trenner pipettes and counting chambers were standardized by the United States Bureau of Standards. The hemoglobin determinations were made by the oxyhemoglobin method of Evelyn.<sup>17</sup> Both wet and dry films were stained with brilliant cresyl blue for counting reticulocytes.

\*Initial attempts to recover the A.P.A. factor from urine by absorbing it on Darco g50 and eluting the Darco with ammoniacal alcohol were unsuccessful.

†Eli Lilly and Company, Indianapolis, Ind.

‡Wilson Laboratories, Division of Wilson & Co., Inc., Chicago, Ill.

Test subjects were patients with pernicious anemia in relapse who were shown to have the peripheral blood and bone marrow changes characteristic of the disease and a histamine refractory achlorhydria. They were given a meat-free diet and were observed for a control period of at least five days before assays were begun.

## RESULTS

1. *Proof That Added A.P.A. Factor Can Be Recovered From Urine.*—The first two extracts were made from urine to which liver extract had been added directly. They were assayed to prove that the extraction method permitted the recovery of A.P.A. factor. Daily intramuscular injections of 0.5 c.c. of Extract

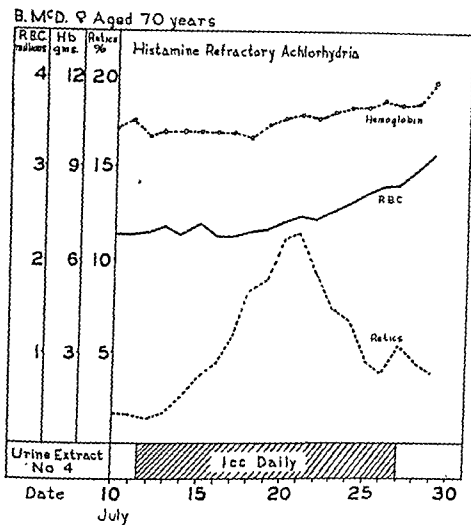


Fig. 1.—Hematologic response induced in a patient with pernicious anemia by an extract made from urine to which liver extract had been added.

1 diluted with 4.5 c.c. of sterile water were given for eight and nine days, respectively, to two test subjects (Table I, R. M. and K. N.). In the first of these (R. M.), a submaximal reticulocyte response of 17.2 per cent developed on the eleventh day. The occurrence of cystitis with accompanying fever (as high as 39.4° C.) during the assay period may well have prevented the reticulocytosis in this patient from reaching the level of 24 per cent, which would have been expected<sup>18</sup> had the therapeutic effect been maximal. The second subject showed the maximum expected reticulocyte rise and an increase in the erythrocyte level of a million cells. In all probability, therefore, each 0.5 c.c. of this extract contained at least 1 U.S.P. unit of the A.P.A. factor. Since the total volume of the extract was 27 c.c., at least 54 U.S.P. units of the original 100 units added to the urine were recovered.

Extract 2 was given intramuscularly in daily doses of 1.0 c.c. (diluted with 2 c.c. of sterile water and 1 c.c. of 1 per cent novocain) for seventeen days to a third test patient (Table I and Fig. 1, B. McD.). A reticulocytosis of 11.4

TABLE I. ASSAYS OF URINE EXTRACTS FOR ANTIPERNICIOUS ANEMIA FACTOR MADE-ON PATIENTS WITH PERNICIOUS ANEMIA IN RELAPSE

SUBJECTS USED FOR ASSAY				HEMATOLOGIC DATA			
				TYPE OF EXTRACT DAILY DOSE (INTRAMUSCULAR)	DAY AFTER START OF THERAPY	R.B.C. (MILLIONS)	HB. (GM.)
PATIENT	SEX	AGE (YR.)					RETICULOCYTES (%)
1. Assay of urine extracts prepared after 100 U.S.P. units of liver extract had been added to 1 liter of urine	R. M.	F	62	0.5 c.c. Extract 1 for 8 days*	0	1.75	8.9
					6	1.64	8.3
					11	1.95	17.2
				50 U.S.P. units of liver extract for 6 days†	0	1.95	17.2
					4	1.97	14.1
2. Assay of extracts prepared from urine collected for twenty-four hours after liver extract had been given parentally	K. N.	F	61	0.5 c.c. Extract 1 for 9 days	4	2.25	24.3
					6	2.82	9.0
					13	2.82	9.0
					19	1.80	0.8
					19	1.41	3.4
3. Assay of extracts prepared from urine collected for twenty-four hours after liver extract had been given parentally	B. McD.	F	70	1.0 c.c. Extract 2 for 17 days	0	2.00	27.1
					5	2.41	8.7
					11	2.48	11.0
					19	3.16	5.3
					19	2.72	1.7
4. Assay of extracts prepared from urine collected for twenty-four hours after liver extract had been given parentally	E. G.	M	47	2.0 c.c. Extract 3 for 9 days	0	2.31	10.5
					6	2.41	10.2
					11	2.48	11.4
					19	3.16	4.0
					19	2.72	1.2
5. Assay of extracts prepared from urine collected for twenty-four hours after liver extract had been given parentally	P. W.			1 U.S.P. unit of liver extract† for 15 days; then 100 U.S.P. units each day for the following 5 days	0	1.39	5.1
					6	1.47	5.3
					6	1.39	5.5
					9	1.30	5.3
					9	1.30	5.3
6. Assay of extracts prepared from urine collected for twenty-four hours after liver extract had been given parentally				1.0 c.c. Extract 4 for 11 days	0	1.30	8.2
					6	1.30	5.5
					12	1.50	6.3
					26	2.49	9.5
					46	3.84	11.3
7. Assay of extracts prepared from urine collected for twenty-four hours after liver extract had been given parentally				2 U.S.P. units of liver extract† for 12 days†	0	4.84	13.4
					11	4.84	13.4
					11	2.60	10.1
					4	2.60	10.3
					8	2.66	10.1
8. Assay of extracts prepared from urine collected for twenty-four hours after liver extract had been given parentally					11	2.62	9.6
					11	2.62	9.6
					11	2.64	9.8
					0	2.64	10.2
					4	2.64	10.7
9. Assay of extracts prepared from urine collected for twenty-four hours after liver extract had been given parentally					8	2.86	12.3
					12	3.19	14.9
					20	3.70	14.7
					41	4.58	14.7
					41	4.58	14.7

\*Patient developed urinary tract infection on second day of therapy; treated with sulfadiazine.

†Commercial liver extract made by the Lederle Laboratories, Inc., Pearl River, N. Y.; 10 U.S.P. units per cubic centimeter.

‡Commercial liver extract made by the Wilson Laboratories, Division of Wilson &amp; Co., Inc., Chicago, Ill.; 10 U.S.P. units per cubic centimeter.



per cent occurred on the eleventh day and the erythrocytes increased from 2.3 to 3.16 million cells per cubic millimeter. This response may also be interpreted as indicating that at least 1 U.S.P. unit of active principle was present per cubic centimeter. Since the total volume of the extract was 30 c.c., one can only conclude that at least 30 of the 100 units added to the urine before extraction were recovered. No attempt was made to refine the assay further in an attempt to make a quantitative estimate of the recovery.

2. *Absence of Detectable A.P.A. Activity in Extracts Prepared From Urine of Subjects Who Had Received Liver Extract Parenterally.*—Extracts 3 and 4 were prepared from urine excreted by two subjects during the twenty-four hours immediately following the last of three and five daily injections of 100 U.S.P. units of liver extract. One of these subjects was a healthy man. The second was a patient with pernicious anemia. Selection of this latter patient was made in order to eliminate the possibility that persons with this disease

F.W. 9 Aged 62 years

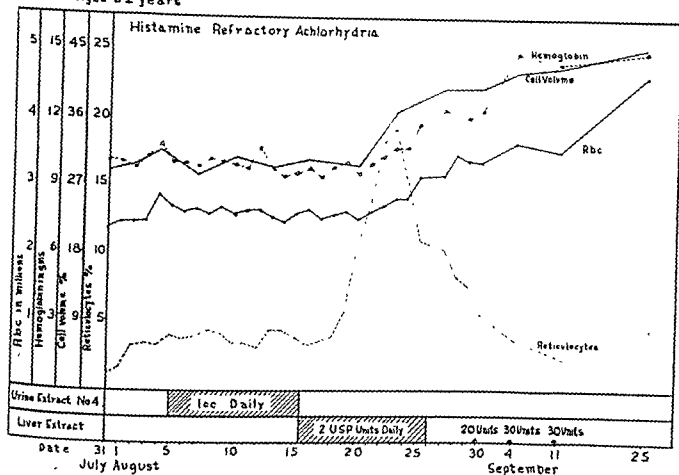


Fig. 2.—Absence of detectable A.P.A. activity in extract prepared from urine of subject who had received liver extract parenterally.

might behave differently from normal individuals in their ability to excrete A.P.A. factor. Neither extract stimulated a reticulocyte response. Two cubic centimeters of Extract 3, diluted with 8 c.c. of sterile water, were injected intramuscularly each day for nine days into a patient with untreated pernicious anemia (Table I, E. G.). No significant change occurred in either the reticulocyte or red blood cell levels. From the tenth to the twenty-fifth day, 1 U.S.P. unit of a commercial liver extract\* was administered intramuscularly each day; the reticulocytes rose to 25 per cent. The total volume of Extract 3 was 40 c.c. Each daily dose, therefore, corresponded to 5 per cent of the total volume. If as few as 20 U.S.P. units had been excreted, and the recovery had been only 50 per cent, some reticulocyte rise should have occurred. In the absence of any effect, it seems reasonable to conclude that less than this amount was excreted by the kidneys.

\*Wilson Laboratories, Division of Wilson & Co., Inc., Chicago, Ill.

Extract 4 in daily doses of 1 c.c. failed to produce a hematologic response in one test subject (Table I and Fig. 2, F. W.). This test patient, however, did respond characteristically when she received 2 U.S.P. units of liver extract per day. The subject from whom urine was collected for preparation of this extract was a man with pernicious anemia who had served as a test patient for assay of Extract 3. After the diagnosis and his ability to respond normally to administration of liver extract had been established, he was given 100 U.S.P. units of liver extract on each of five days. Urine was collected during the twenty-four hour period which immediately followed the last injection.

These observations served to establish two things: (1) With the methods used, no detectable A.P.A. activity could be extracted from urine excreted within twenty-four hours after a relatively large dose of liver extract; (2) the data rule out the possibility that the reticulocyte responses obtained with Extracts 1 and 2 were nonspecific.

#### DISCUSSION

If the A.P.A. factor is a relatively small molecule, it is rather surprising that after large doses were given parenterally detectable amounts failed to appear in the urine. The result, however, is compatible with the clinical experience summarized in the introductory paragraph which indicates that a great proportion of the active principle is stored even when it is given in large amounts. Nevertheless, from Wilkinson's observations<sup>1</sup> that small amounts of A.P.A. substance can be recovered from large volumes of urine, one would logically expect that after parenteral administration of liver extract there might temporarily be an increased urinary output of A.P.A. factor.

In the clinical management of patients with pernicious anemia, however, there are many reasons for being cautious about recommending that the interval between injections be lengthened to more than six or eight weeks; for instance, the desirability of observing the patients at regular intervals, the fear that the antianemia factor may not be identical with the substance required for the control and prevention of combined system disease, the possibility that some intercurrent disease might increase the requirement for A.P.A. factor so as to disrupt calculations as to requirement, and the probability that sensitization to liver extract occurs more frequently as intervals between injections are prolonged. The results of the present study would suggest that large amounts of liver extract injected at widely spaced intervals might be stored efficiently.

#### SUMMARY

1. A method has been described by which at least 50 per cent of the A.P.A. factor can be extracted from urine containing a known amount of this substance.

2. No A.P.A. activity could be detected in the urine excreted by two subjects during the twenty-four hours immediately following the last of from three to five daily injections of 100 U.S.P. units of liver extract.

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# THE ACTIVITY OF SYNTHETIC *LACTOBACILLUS CASEI* FACTOR ("FOLIC ACID") AS AN ANTIPERNICIOUS ANEMIA SUBSTANCE

## I. OBSERVATIONS ON FOUR PATIENTS: TWO WITH ADDISONIAN PERNICIOUS ANEMIA, ONE WITH NONTROPICAL SPRUE AND ONE WITH PERNICIOUS ANEMIA OF PREGNANCY

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ADMINISTRATION of the synthetic *Lactobacillus casei* factor of Angier and co-workers<sup>1</sup> stimulates a reticulocyte response and an increase in the red blood cell count in patients with macrocytic anemia in relapse.<sup>2a, b, c</sup> Spies and associates<sup>2a</sup> observed these hematologic effects in nine patients, four of whom were given the *L. casei* factor by mouth and five by parenteral routes. The oral dose was either 100 or 150 mg. daily; one patient was given a single intravenous injection of 20 mg., another was given 50 mg., while three patients were given daily injections of 20 mg. each. These patients are described only as having "macrocytic anemia in relapse." Data were not reported which would make it possible to determine whether they had nutritional macrocytic anemia or Addisonian pernicious anemia. In patients seen at the Nutrition Clinic of the Hillman Hospital, it frequently is difficult to differentiate, without long periods of study, between these two types of macrocytic anemia.<sup>3</sup> Darby and Jones<sup>2b</sup> obtained satisfactory remissions in two patients with sprue who were given 15 mg. of the same material intramuscularly per day. More recently Spies and co-workers have noted similar effects in several patients with tropical sprue.<sup>2c</sup> The findings described in the present communication confirm and extend these observations. Satisfactory hematologic and clinical remissions have been induced by the administration of synthetic *L. casei* factor to two patients with Addisonian pernicious anemia, to one patient with nontropical sprue, and to one patient with pernicious anemia of pregnancy. Data are presented which indicate that the compound has antipernicious anemia rather than extrinsic factor activity.

Before these results are presented, however, use of the term "*Lactobacillus casei* factor" instead of "folic acid" should be explained. "Folic acid" is the name given by Mitchell, Snell, and Williams<sup>4</sup> to a substance, obtained in nearly pure form from spinach, which supports growth of two organisms commonly used in microbiologic investigation: *L. casei* and *Streptococcus lactis R* (now termed *S. faecalis*). Its potency for *S. lactis R* was used as a guide in purification procedures, and "folic acid" is defined specifically as a growth factor for that organism.<sup>5</sup> A number of other substances with similar physiologic properties have more recently been isolated from liver, yeast, or other sources; at least three of these substances have been obtained in crystalline form. Some of these compounds either will not support the growth of *S. lactis R*, or affect its

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growth less markedly than that of *L. casei*, and it is, strictly speaking, incorrect to designate them as "folie acids." "*L. casei* factor (s)" is a more appropriate, but less euphonious, generic term.<sup>8</sup>

More specifically, the synthetic material supplied to us is referred to as *L. casei* factor because it is reported to be identical with a crystalline preparation isolated from liver by Stokstad.<sup>8a</sup> This substance, which was termed "liver *L. casei* factor," was equally potent as a growth factor for the two bacteria mentioned and appeared to be the same as crystalline vitamin B<sub>12</sub> extracted from liver by Pfaffner and co-workers.<sup>9</sup> On the basis of data from absorption spectra, Hutchings and his associates concluded that it could not be identical with "folie acid" prepared from spinach.<sup>10</sup> The terminology is confusing and the relation of these several factors to each other is not entirely clear. For a more complete statement of the problem, reference should be made to the recent review by Daft and Sebrell.<sup>6</sup> The chemical structure of the "liver *L. casei* factor" has not been announced, although its synthesis has been reported by Angier and associates.<sup>1</sup>

It is not surprising that the synthetic *L. casei* factor has been found to have antianemic properties in man; experiments have abundantly demonstrated that identical or closely related nutritives influence the metabolism of bone marrow in experimental animals. For instance, crystalline vitamin B<sub>12</sub> prevents<sup>9</sup> the Hogan-Parrott type<sup>10</sup> of nutritional anemia in chicks. Complete remissions in three monkeys with nutritional cytopenia (formerly termed vitamin M deficiency and characterized by anemia, leucopenia, and thrombocytopenia) treated with a highly purified preparation of *L. casei* factor<sup>8</sup> are described in a recent report.<sup>11</sup> Rats fed a highly purified diet containing all the other known components of the vitamin B complex and one of the sulfonamide drugs develop hypocellularity of the bone marrow, leucopenia, granulocytopenia, and an anemia.<sup>12a</sup> All of these manifestations are corrected by liver extracts known to contain *L. casei* factor, by crystalline vitamin B<sub>12</sub>, and by a crystalline *L. casei* factor.<sup>8,12b</sup>

Although consideration was given by Wright and Welch<sup>12c</sup> to a possible relationship of "folie acid" to pernicious anemia, earlier attempts to discover therapeutic effectiveness of "folie acid" or of *L. casei* factor for anemias or leucopenias in man yielded indifferent results. The present authors assayed several concentrates of "folie acid" in 1943 for extrinsic factor (Castle) activity. These concentrates were prepared either from grass juice<sup>12d</sup> or from spinach. Patients on whom the assays were done had classical hematologic and clinical manifestations of pernicious anemia, and were fed a meat-free diet. To the first subject, 500 mg. of a crude concentrate<sup>12e</sup> from grass juice were given each day for fifteen days together with 125 c.c. of normal human gastric juice. Reticulocytes began to increase on the fifth day of this period and reached a maximum of 25.6 per cent on the fourteenth day; meanwhile the red blood cell count rose from 1.3 to 1.7 million cells per cubic millimeter. That the response had been submaximal was indicated by the fact that an additional small reticulocyte rise occurred when 1 oral U.S.P. unit of liver extract was given daily beginning with the sixteenth day. This result seemed to indicate that such concentrates might contain some extrinsic factor activity. Although several other patients were given concentrates without comparable responses, some of these patients were found subsequently to respond very poorly to oral liver extracts. Among the preparations studied was a more highly purified sample of "folie acid," prepared from spinach and kindly presented to us by Dr. R. J. Williams of the University of Texas. Fifteen milligrams of this material given daily for eleven days, together with 125 c.c. of normal human

gastric juice, supplied an amount of "folic acid" equivalent to that contained in the cruder concentrate given previously. In this dosage, the preparation failed to produce a significant rise in reticulocytes or erythrocytes (the red cell count initially was about 1.4 million per cubic millimeter). When the patient was given 1 U.S.P. oral unit of liver extract daily, the reticulocytes, by the fifteenth day, had risen to 18.5 per cent and the erythrocyte count was 2.37 million per cubic millimeter.

This patient had previously been given for ten days, without any significant response, daily intramuscular doses of 5 c.c. of a crude preparation of *L. casei* factor, containing in each dose from 0.06 to 0.12 mg. of microbiologically active material.

Negative results were obtained by Castle and his associates<sup>13</sup> when vitamin-free casein and all the crystalline members of the vitamin B complex including "folic acid and folic acid concentrate" in amounts of 2.3 to 3.6 mg. daily were added to normal gastric juice and fed daily to patients with pernicious anemia in relapse.

Sharp, Vonder Heide, and Wolter<sup>14</sup> gave daily doses of a yeast concentrate said to contain 0.6 to 1.5 mg. of vitamin B<sub>12</sub> to ten patients with anemias that had proved refractory to liver and iron. They obtained "an appreciable increase in the hematocrit but only slight changes in other erythropoietic phenomena." Watson and his associates<sup>15</sup> gave 5 mg. of *L. casei* factor<sup>16</sup> daily by mouth for six days to eight patients with refractory anemia, and one patient with leucopenia persisting after sulfonamide therapy; no therapeutic effect was obtained in any case. Elevations of the leucocyte count, however, were produced by similar doses administered to 7 patients who had developed leucopenia following roentgen-ray therapy; the elevations in the white counts did not persist after the *L. casei* factor was discontinued.

#### METHODS AND MATERIAL

The hematologic techniques used in this investigation have been described in an earlier publication.<sup>3</sup> Fat determinations on stool specimens were performed according to the method described by Hanes.<sup>16</sup>

All patients in the study were hospitalized and all, with the exception of the woman with nontropical sprue, were fed a meat-free diet. Observations were made during control periods of two to five days or more and complete blood counts were made daily. Changes in the peripheral blood and bone marrow in each instance were those characteristic of pernicious anemia in relapse. Results of gastric analyses and fluoroscopic studies of the gastrointestinal tract are recorded in case summaries at the end of this paper. Two healthy laboratory workers served as control subjects; one took 100 mg. of synthetic *L. casei* factor orally each day for 10 days, the other was given daily intravenous injections of 20 mg. for a similar period.

When the synthetic compound was given orally, it either was placed in capsules or was suspended in water. For parenteral administration, 100 mg. of synthetic *L. casei* factor and 100 mg. of sodium bicarbonate were added to 20 c.c. of freshly distilled water, placed in a serum vial, and autoclaved for 15 minutes at 15 pounds of pressure. The solution was then clear. New solutions were prepared at least once every three days. Sterilization through a Seitz filter was abandoned because of uncertainty concerning the amount of absorption of the vitamin by the filter. All weighings were accurately made on an analytical balance. Injections were given rapidly and no reactions were observed.

## RESULTS

1. *Oral Administration of Synthetic L. casei Factor ("Folic Acid") to Two Patients With Addisonian Pernicious Anemia and to One Normal Control Subject.*—The synthetic *L. casei* factor was given orally to two patients with true pernicious anemia because it was felt that a therapeutic effect would not be induced if the material behaved as Castle's extrinsic factor, unless normal human gastric juice were administered at the same time. On the other hand, if the material stimulated a remission without added gastric juice, the result would constitute presumptive evidence that the compound possessed antipernicious anemia factor activity. The first patient treated (M.G.) was given 100 mg. of the synthetic preparation orally each day for ten days. Her initial red blood cell level was approximately 1.2 million cells per cubic millimeter. On the third day of therapy she experienced the sense of well-being and the increased appetite which usually occurs with liver therapy at this time. On the following day her reticulocytes began to rise and a peak value of 40 per cent was reached on the seventh day (Fig. 1). Her red blood cells began to increase in number on about the seventh day and the count rose rapidly until a level slightly over 3.0 million cells was attained. At this point the rise stopped (therapy was discontinued after the tenth day) and a plateau has existed for nearly two weeks. Further therapy was intentionally withheld in order that we might determine how high the erythrocyte level would go following ten days of dosage (1 gram).

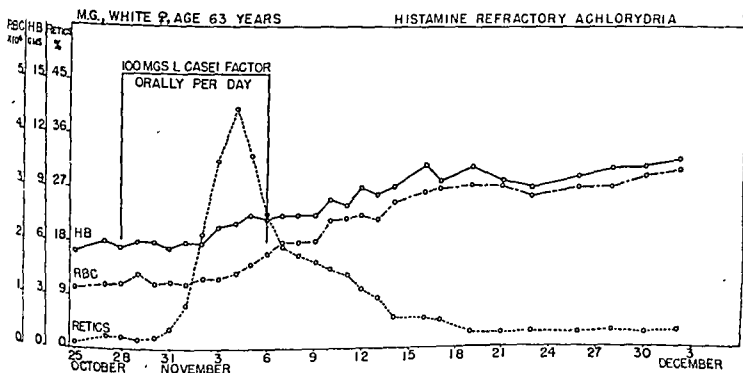


Fig. 1.—Hematologic response of a patient with Addisonian pernicious anemia to the oral daily administration of 100 mg. *L. casei* factor.

To the second patient (E.K.), a daily oral dose of 30 mg. was given for fourteen days. This woman was 78 years of age and had a red blood cell count of only 0.7 to 0.95 million cells per cubic millimeter at the time of her admission to the St. Louis City Hospital. Because of her critical condition, a transfusion of 500 c.c. of citrated whole blood was given. This increased her erythrocyte count to 1.4-1.5 million cells per cubic millimeter. The reticulocytes began to increase on the fourth day of therapy; a maximum of 44.5 per cent occurred on the eighth day (Fig. 2). The rise in the red cell count began on the sixth day and was as rapid as is usual with liver therapy. This response is particularly noteworthy because the patient developed bronchopneumonia and a

decubitus ulcer on the sixth day; she was given penicillin and was digitalized with Digoxin injected intravenously. She was critically ill for three days but is now greatly improved. The irregularity of the erythrocyte curve from the sixth to the tenth days is probably attributable in part at least to the degree of dehydration present during that period. On the fourteenth day, her red blood cell count was 2.5 million per cubic millimeter. Attention is also directed to the fact that the original leucopenia and thrombocytopenia were corrected by the *L. casei* factor. During the period of pneumonia, there occurred a leucocytosis of 22,800 cells per cubic millimeter.

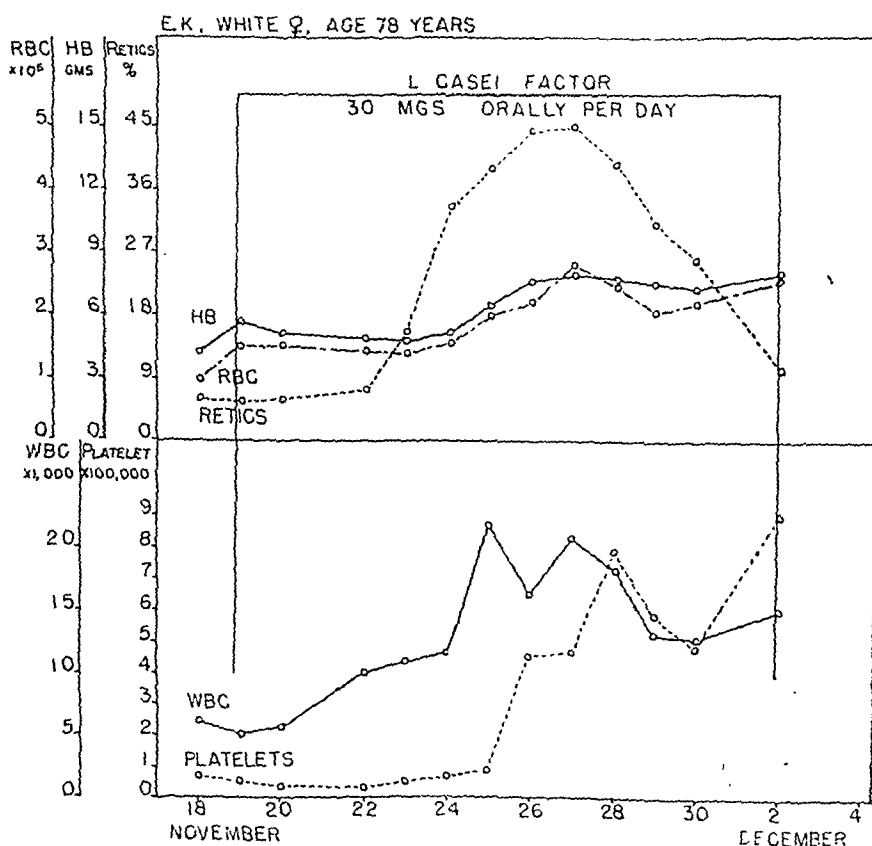


Fig. 2.—Hematologic response of a patient with Addisonian pernicious anemia to the daily oral administration of 30 mg. *L. casei* factor.

The normal control subject, who ingested 100 mg. of the synthetic *L. casei* factor daily for ten days, showed no change in reticulocytes, red cells, hemoglobin, leucocytes, or platelets during three weeks of observation. She noted no symptoms and no subjective changes.

2. *Intravenous Administration of Synthetic L. Casei Factor ("Folic Acid") to One Patient With Nontropical Sprue, to One Patient With Pernicious Anemia of Pregnancy, and to One Normal Control Subject.*—Twenty milligrams of synthetic *L. casei* factor were injected intravenously each day for ten days to J.S., a woman 54 years of age who had all the classical manifestations of nontropical sprue. The oral glucose tolerance curve was flat while the intravenous tolerance test was normal, stool fat varied from 50 to 75 per cent of dry weight, the serum calcium was low while alkaline phosphatase was high, and weight loss had been extreme. Following the initial ten-day period, 40 mg. were injected



every other day for an additional two weeks. The number of reticulocytes began to increase on the third day of therapy and a value of 30.2 per cent was attained on the seventh day, in spite of the fact that her initial red blood cell count was 2.6 million cells (Fig. 3). The authors have never seen a reticulocytosis of this degree following the parenteral administration of liver extract to patients with pernicious anemia who had comparable red blood cell counts. Subjective clinical improvement occurred, but this change was not dramatic; the amount of fat in the stool did not decrease until after the fat in her diet had been decreased from about 200 to 70 grams per day. The erythrocyte count rose to approximately 3.5 million cells, at which level the mean corpuscular volume had become normal (88 cubic microns) and the mean corpuscular hemoglobin concentration had decreased to 28 per cent. It is of interest that this patient had a hypochromic microcytic anemia four months previous to the present hospitalization (see case summary). Leucocytes began their climb to normal levels on the third day; platelets began to increase on the sixth day. Values for both these formed elements are now normal one month after institution of therapy. The patient has gained 6 pounds in weight within four weeks; her oral glucose tolerance test still shows poor absorption.

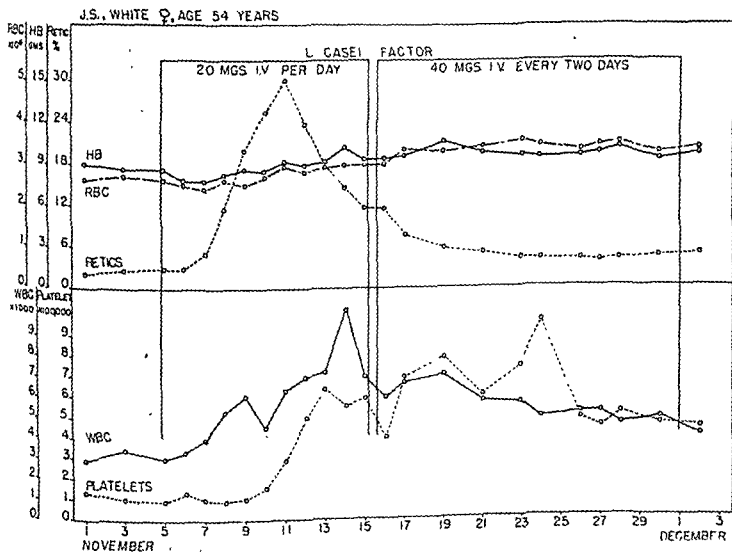


Fig. 3.—Hematologic response produced in a patient with a macrocytic anemia of nontropical sprue by the daily intravenous administration of 20 mg *L. casei* factor.

The patient with pernicious anemia of pregnancy (M. W.) is a young woman 32 years of age who had developed a similar anemia during each of four previous pregnancies. Liver therapy was given when her third and fourth children were born but she was not carefully followed; she remained subjectively well between pregnancies. She ate liver ordinarily about twice a month but took no additional amounts. At the time of parturition on Oct 24, 1945, in another hospital, she was found to have a red blood cell count of only 1 million cells. Transfu-

sion was refused; liver extract was not given; and she signed out of the hospital under protest. Because she was totally unable to do her work and was very weak, she asked to be admitted to the St. Louis City Hospital on Nov. 11, 1945. Her red blood cell count was still only 1.1-1.2 million cells per cubic millimeter. On Nov. 18, 1945, and for each of the following nine days, she was given 20 mg. of synthetic *L. casei* factor intravenously. Her subjective improvement was marked on the third day, the number of reticulocytes had begun to increase by that time and had attained a peak value of 48.2 per cent on the seventh day of therapy. The rise in red blood cells has been dramatic and a level of 3.0 million per cubic millimeter was observed on the fifteenth day (Fig. 4). She then felt very well and was discharged from the hospital. This woman never had a leucopenia. Her platelets did not begin to rise from their initially low level until the ninth day.

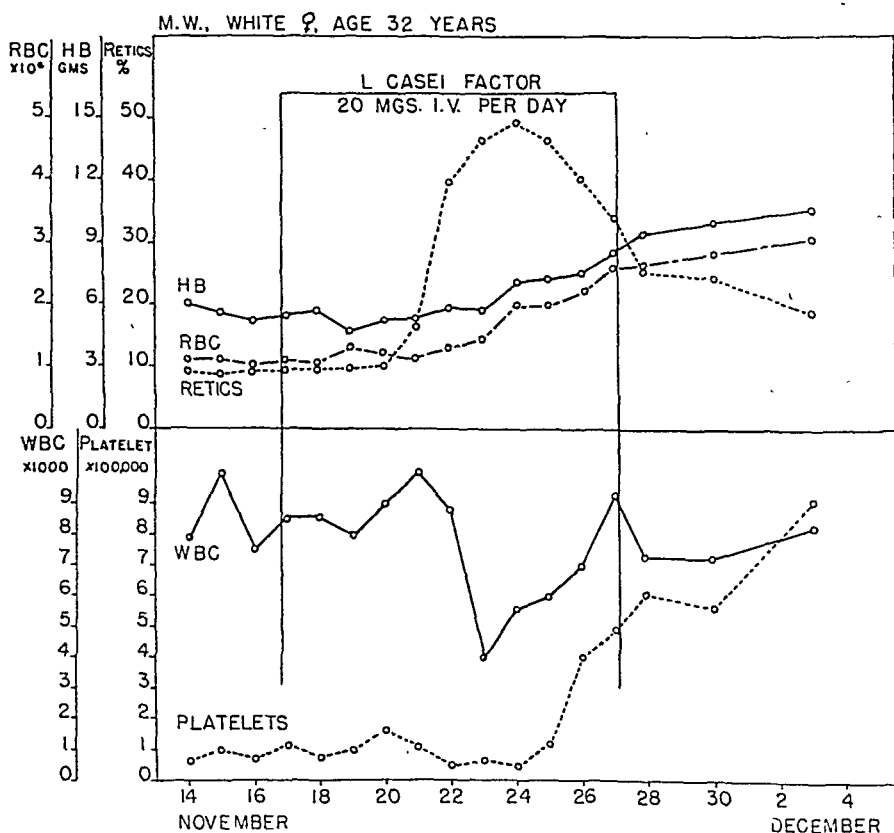


Fig. 4.—Hematologic response of a patient with pernicious anemia of pregnancy to the daily intravenous administration of 20 mg. *L. casei* factor.

The normal control subject, who was given a daily dose of 20 mg. of the material intravenously for ten days, has been followed for three weeks. No change occurred in any of the cellular elements of the blood and no symptoms or exhilaration were experienced.\*\*\*\*

#### DISCUSSION

The fact that synthetic *L. casei* factor, administered orally without gastric juice and intravenously, induced hematologic and clinical remissions in these patients, suggests that the compound has antipernicious anemia rather than extrinsic factor activity. Caution must be used in drawing final conclusions

about this differentiation, however, for two reasons. Wintrobe<sup>17</sup> has shown that good therapeutic responses may be induced in patients with Addisonian pernicious anemia by the daily oral feeding of 1 to 2 grams of brewer's yeast per kilogram of body weight. If yeast contains only extrinsic factor activity, as has been thought, then the small amounts of intrinsic factor present in the gastric secretions of patients with pernicious anemia form enough erythrocyte maturation factor to be therapeutically effective. The daily doses of 30 mg. or of 100 mg. of synthetic *L. casei* factor may possibly be comparable in extrinsic factor activity to the large doses of yeast used by Wintrobe. Second, no extrinsic factor preparation has ever been available in pure enough form to permit its use parenterally. There is, therefore, no information available as to what effect Castle's extrinsic factor would have were it to be injected intravenously.

Even if synthetic *L. casei* factor has antipernicious anemia factor activity, as the available evidence seems to indicate, it has not been established that this factor is identical with the antipernicious anemia factor in liver extracts. The parenteral doses used were 20 mg. daily; investigators who have fractionated liver extracts in an attempt to isolate the active principle have obtained good responses with much smaller quantities of material. Strandell, Poulsson, and Schartum-Hansen,<sup>18</sup> for instance, produced a good hematologic effect with 3 injections of a fraction which contained only 0.7 mg. of material per injection. Alexander<sup>19</sup> was able to obtain a reticulocytosis of 114 per cent in a patient with pernicious anemia, whose original red blood cell level was 1.9 million cells per cubic millimeter, when 0.035 mg. of a fraction was given per day. Furthermore, Wright and Welch<sup>12c</sup> found that a commercial liver extract (Armour; 15 U.S.P. injectable antipernicious anemia units per cubic centimeter) contained only 14 mg.-units of microbiologically active "folic acid" (*L. casei* assay) per cubic centimeter, or about 0.25 to 0.5 micrograms of *L. casei* factor per unit of antipernicious anemia activity. Clark<sup>20</sup> has reported that various commercial liver extracts contain, per U.S.P. unit, only 0.02 to 3.67 micrograms of *L. casei* factor. A liver extract made by the Wilson Laboratories (2 U.S.P. units per cubic centimeter) has only 0.38 micrograms of "folic acid" per unit.<sup>21</sup> In terms of this latter preparation, the intramuscular administration of 1 U.S.P. unit daily for 10 days would provide only 3.8 micrograms; with the 20 mg. dose of synthetic *L. casei* factor, we actually gave 200,000 micrograms during a similar period. Although the amounts of conjugated or "potential" *L. casei* factor in these liver extracts are not known, it is evident that synthetic *L. casei* factor must be proved effective in much smaller doses before it can be accepted as being identical with the antipernicious anemia factor in liver.\*\*\*\*

Rat liver has been found to contain only about 10 micrograms or less of free *L. casei* factor per gram.<sup>22</sup> The daily ingestion of 250 to 300 grams of pork or beef liver will usually provide a satisfactory therapeutic effect for patients with Addisonian pernicious anemia. Since pork and beef liver contain similar amounts of microbiologically active "folic acid," only 2.5 to 3.0 mg. of free "folic acid" would be supplied by 250 to 300 grams. Under these circumstances, one would expect that if the *L. casei* factor were the antipernicious anemia factor present in liver, the patient who received 100 mg. of the synthetic factor daily for ten days should have had enough active principle to raise her red cell level to 5 million rather than to only 3 million cells. It may be, as SubbaRow, Hastings, and Elkin<sup>23</sup> suggest, "that the activity of material effec-

tive in the treatment of pernicious anemia rests upon a certain type of compound or linkage common to more than one substance, as is believed to be the case of some of the vitamins."

On the other hand, the data available on the "folic acid" content of various tissues and foods have been obtained through the use of microbiological assays and it is now known that many materials contain relatively large amounts of *L. casei* factor in a combined form (milk: Welch and Wright,<sup>24</sup> Wright and co-workers<sup>25</sup>; yeast: Binkley and associates,<sup>26</sup> Mims, Totter, and Day<sup>27</sup>). Mal-lory, Mims, Totter, and Day<sup>28</sup> and Binkley and his co-workers<sup>29</sup> have studied the enzymatic release of microbiologically active material from the combined or conjugated form, and Laskowski and associates<sup>30</sup> have partially purified an enzyme found in chicken pancreas (and other tissues) which accomplishes this release of the free *L. casei* factor. It is not unlikely that the amount of "potential" *L. casei* factor ingested in the diet is very much larger than microbiological assays have indicated. It is conceivable that in certain pathologic conditions there is a failure to utilize or to absorb this "potential" *L. casei* factor, as a result of which anemia of the macrocytic type might develop. Possibly the intrinsic factor of Castle is concerned with the release in man of the *L. casei* factor from its combined form.

The significance of and the variations in the synthesis of the *L. casei* factor by the intestinal bacteria, so extensively studied in the rat, must now for obvious reasons be thoroughly investigated in man.

Since the patients reported by Spies and his co-workers<sup>2</sup> were said only to have macrocytic anemia in relapse, and no differentiation was made between nutritional macrocytic anemia and Addisonian pernicious anemia, the two patients with true pernicious anemia here reported are the only two so far known to have responded specifically to synthetic *L. casei* factor. The woman with pernicious anemia of pregnancy is also of special interest. Patients with this disease usually respond to parenterally administered liver extract, but Watson and Castle<sup>30</sup> have recently observed two such patients who responded to oral but not parenteral therapy. This observation raised the question as to whether the pathogenesis of pernicious anemia of pregnancy is always the same as that of Addisonian pernicious anemia. It is of interest, therefore, that *L. casei* factor administered parenterally was capable of inducing a remission in at least this one subject.

Finally it should be pointed out that in none of the cases so far studied has the synthetic *L. casei* factor been given for a sufficiently long period to determine whether relapses will occur during continued administration of the vitamin. There has not been sufficient time to determine what effect *L. casei* factor has on the neurologic manifestations of pernicious anemia.

#### SUMMARY AND CONCLUSIONS

Clinical and hematologic remissions were induced in two patients with Addisonian pernicious anemia by the daily oral administration for ten days of 30 mg. and of 100 mg. of synthetic *L. casei* factor ("folic acid"), respectively. One patient with macrocytic anemia of nontropical sprue and one patient with pernicious anemia of pregnancy responded in a similar manner when given daily 20 mg. of the preparation intravenously.

Reasons are given for interpreting these results as indicating that *L. casei* factor possesses antipernicious anemia factor activity. It is pointed out, how-

ever, that the material must be proved effective parenterally in much smaller doses, and over a much longer period of time, before it can be accepted as being closely similar in action to (or identical with) the effective principle present in liver extracts.

#### CASE REPORTS

M. G., white female, 63 years of age. This woman was admitted to the St. Louis City Hospital on Oct. 24, 1945 with the chief complaints of progressive pallor, weakness, and weight loss during the previous three months. She had been a ward attendant at the City Hospital for many years. During the summer, she noticed that the work she previously had done without difficulty was becoming increasingly hard. As a result, she decided not to return to her job after her vacation in August. Even with very little work to do at home, however, she was increasingly aware of her weakness and her family observed that she was becoming pale. There was a weight loss of approximately 13 pounds from midsummer to October. Five days before admission she had an emesis of green colored vomitus which contained no food particles, she felt dizzy, and had a sense of sub-sternal oppression. The latter symptom recurred whenever she moved about her home and disappeared within a few minutes after she would lie down. Melena had never been observed. She did not complain of paresthesias.

The patient has been in reasonably good health during most of her life. Her hair began to turn white at the age of 55 years. Her only previous hospitalization had been for an upper respiratory infection in February, 1945; at that time her red blood cell count was normal.

Physical examination showed the patient to be a rather small, blue-eyed, white-haired elderly woman who lay very quietly in bed and slept almost constantly except when she was disturbed. Her skin and mucous membranes were pale and a distinct lemon yellow color was present. Vessels of her retina showed arteriosclerotic changes. She was edentulous and the sides of her tongue were smooth. There was a dorsal kyphosis and fine moist rales were heard at both bases. The area of cardiac dullness extended 10 cm. to the left of the midsternal line and a soft systolic murmur, loudest at the base, was heard over the whole precordium. Cardiac rhythm was made irregular by frequent premature systolic contractions. Blood pressure 120/52. Liver and spleen were not palpated; no abdominal masses were felt. Pelvic and rectal examinations were essentially negative. There was no significant degree of lymph node enlargement. A few small purpuric areas were present over the dorsum of the hands and forearms. All deep reflexes were normally active and of equal intensity on the two sides. There was no detectable disturbance of position or of vibratory sense.

Urinalysis was within normal limits. No occult blood or parasites were found in the feces. The Kahn test was negative. The initial blood studies showed: red blood cell count 1,170,000 cells per cu. mm., hemoglobin 5.4 Gm., reticulocytes 0.7 per cent, hematocrit 18 per cent, white blood cell count 3,400. There was marked anisocytosis and poikilocytosis of the red blood cells with a mean corpuscular volume of 153 cubic microns and a mean corpuscular hemoglobin concentration of 33 per cent. Leucocytic differential was within normal limits but the polymorphonuclear leucocytes were hypersegmented. Bone marrow removed by sternal aspiration appeared hyperplastic, particularly for erythroid elements. There was a distinct increase in the percentage of early erythroblasts and megaloblasts. Icterus index 16. Cephalin cholesterol flocculation test two plus. The I.V. hippuric acid test resulted in a urinary excretion of 0.43 Gm. of sodium benzoate within 1 hour. N.P.N. was 22 mg. per cent. Total plasma protein concentration was 6.88 Gm. per 100 c.c. with 4.42 Gm. of albumin and 2.45 Gm. of globulin. A histamine refractory achlorhydria was present.

Roentgenogram of the chest showed evidence of marked peribronchial fibrosis in both lung fields, areas of calcification in the descending aorta, moderate cardiac enlargement, and kyphosis in the midthoracic region. Fluoroscopic study of the gastrointestinal tract revealed no abnormalities except two rounded areas of irregular calcification in the gall bladder region. Electrocardiogram showed the changes of left bundle branch block.

As is stated in the body of the paper, this patient experienced a striking sense of well-being on the third day of therapy with *L. casei* factor. She has now improved markedly in strength, is gaining weight, and no longer experiences sub-sternal oppression when she walks around the ward.

E. K., white female, 73 years of age. This woman is a spinster who lives alone. The history she gives is not entirely coherent and other members of the family are not able to provide much additional information. Her hair had begun to turn white before she was 30 years old. Menopause occurred ten years later. In 1927 or 1928, she noted weakness, tingling in her feet and hands, sore tongue, dyspnea, and slight ankle edema. The physician consulted told her she had an anemia and advised her to eat liver. She improved remarkably and all symptoms other than her paresthesias disappeared. A number of doctors were seen between this time and 1939; some gave her liver extracts but the patient was quite irregular either in reporting to her physicians or in eating liver. In October of 1939, she became nauseated and vomited persistently for several weeks. She became increasingly weak and was soon confined to bed. About the middle of November her sister told her she was jaundiced. There was no hematemesis, no melena. A weight loss of 10 to 20 pounds occurred during this period.

On Nov. 21, 1939, the patient was admitted to the St. Louis City Hospital. She was pale, edentulous; had a smooth, glistening tongue; her liver was palpable 2 cm. below the right costal margin; pitting edema of both feet and legs was detected; the Babinski sign was bilaterally present. Blood pressure 140/60. Her red blood cell count was 1,200,000 cells per cubic millimeter, hemoglobin 5 gm., and white blood cells 4,500. The red cells were macrocytic and varied greatly in size and shape. Bone marrow studies were said to be compatible with a diagnosis of Addisonian pernicious anemia. A histamine refractory achlorhydria was present. Twenty units of liver extract were given parenterally each day. By the seventh day, her reticulocytes had risen to 35 per cent. When she was discharged from the hospital, the red blood cell level had risen to 4,080,000 and the hemoglobin to 14.5 grams. No other therapy had been given. Fluoroscopic study of the gastrointestinal tract revealed no abnormalities.

For several years, the patient returned at regular intervals to the Out-patient Department for injections of liver extract. She felt well and was unusually agile for a woman of her age. In May of 1941, she was readmitted to the City Hospital for several days because of a skin eruption on her legs which was said to be an "eczema." It disappeared after boric ointment was regularly applied. Her red blood cell count at that time was slightly over 4 million cells.

Visits to the Clinic became irregular in 1944 and finally stopped altogether about five months previous to the present hospitalization. She remained well until the end of October when she again became nauseated and began to vomit. She lost her appetite, became progressively weaker, and became bedfast early in November. There was no orthopnea, no dyspnea, but ankles were occasionally swollen. No hematemesis or melena was observed.

Physical examination revealed a lethargic, almost comatose elderly woman who was very pale and who had a slight icteric tint to her sclera and skin. Her temperature was 38.5° C., pulse 92, respirations 36, and blood pressure 120/50 mm. of Hg. Her skin was dry and hot. Tongue was smooth; mouth, edentulous. There were signs of fluid in the right pleural cavity. A moderately loud systolic murmur was audible over the entire precordium. No masses or organs were palpable in her abdomen. Reflexes were normally active; there were no pathologic toe signs; vibratory sense was diminished over both legs.

Urinalysis showed 2 plus protein and a rare granular cast. The hematologic data are summarized on the chart (Fig. 2). Red blood cells were macrocytic and varied greatly in size and shape. The differential white blood cell count was normal except for an occasional myelocyte and for hypersegmentation of the polymorphonuclear neutrophils. Sternal marrow was hyperplastic and showed a striking increase in megaloblasts. Blood Kahn test was negative; the N.P.N. was 32 mg. per cent. Total plasma proteins were 5.2 Gm. per 100 c.c. with 3.3 Gm. of albumin and 1.0 Gm. of globulin. Venous pressure was 122 mm. of saline. Pleural fluid showed a specific gravity of 1.013 and a protein content of 2.85 Gm. per 100 cubic centimeters.

Because of the patient's critical condition, a transfusion of 500 c.c. of citrated blood was given shortly after she entered the hospital. Her temperature returned to normal. The following day administration of 30 mg. *L. casei* factor orally per day was begun. On the sixth day, however, signs of bronchopneumonia developed and a decubitus ulcer formed over her sacral area. She was given 1.0 mg. digoxin intravenously. Administration of penicillin in doses of 40,000 units every 2 hours (intramuscularly) was begun; after 12 hours, the amount was reduced to 20,000 units every 2 hours. Within 72 hours she was markedly improved. Administration of penicillin has now been discontinued. She is eating well, and feels much stronger.

J. S., white female, 54 years of age. During the last four or five years, this woman has had from one to three formed or semiformed, light-colored stools per day. To her knowledge, they have never been foamy. She lost approximately 40 pounds in weight during this period and became progressively weaker. Early in 1945 she noted stiffness of her muscles and generalized pains throughout her body. Shortly thereafter her ankles began to swell but there was no associated dyspnea or orthopnea. She was a patient in the Barnes Hospital from July 2, 1945, to August 24, 1945. At this time it was discovered that she had a hypochromic microcytic anemia with a red cell count of slightly over 4 million cells and a hemoglobin value of approximately 9 grams. Her serum calcium was found to be 7 mg. per 100 c.c., serum phosphorus 3.5 mg. per cent and the alkaline phosphatase 14 Bodansky units. Because of these changes, a diagnosis of osteomalacia was made and she was given large doses of both vitamin D and of calcium. After her discharge from the hospital, she improved steadily but gained very little weight. Since no cause for the development of osteomalacia had been determined, and since it was felt that her improvement had not been satisfactory, she was readmitted to the hospital on Oct. 26, 1945.

Physical examination showed the patient to be an extremely emaciated white woman of middle age who walked slowly with a portable walker. She was comfortable, cooperative, and complained chiefly of the fact that she had been returned to the hospital. Her mucous membranes were moderately pale and her skin showed a slight brownish pigmentation very much like that of a mild sunburn; it was dry and inelastic. There was no significant enlargement of lymph nodes. The tongue was moderately smooth. The heart and lungs were not remarkable. The abdominal wall was very thin so that peristaltic patterns were easily seen; the liver edge was palpable 1 cm. below the right costal margin; no other organs or masses were found. Pelvic examination showed the vaginal mucosa to be atrophic. Rectal examination was negative. Examination of the extremities showed pronounced atrophy of both leg and thigh muscles. Deep reflexes were normally active and equal on the two sides. There were no pathological toe signs and no sensory disturbances were detected.

Urinalysis showed a trace of protein. Stool examinations were repeatedly negative for the presence of occult blood or of parasites. Blood Kahn reaction was negative. Hematologic studies were as follows: red blood cells 2,630,000, hemoglobin 9 grams, reticulocytes 1.2 per cent, white cells 2,850, platelets 129,000. The leucocytic differential was normal. There was definite macrocytosis of the red cells (mean corpuscular volume 114 cubic microns) with anisocytosis and poikilocytosis; the polymorphonuclear neutrophils showed hypersegmentation. Bone marrow removed by sternal aspiration was normally cellular but showed an increase in the percentage of erythroid elements with a definite increase in the number of early erythroblasts and megaloblasts. Total serum protein was 5.9 Gm. per 100 c.c. with 4.0 Gm. of albumin and 1.9 Gm. of globulin. In an oral glucose tolerance test the fasting blood sugar was 69 mg. per cent while the highest value reached following the injection of glucose was only 80 mg. per cent. An intravenous glucose tolerance test the following day showed a fasting blood sugar of 67 mg. per cent; 251 mg. per cent at the end of one-half hour after injection of the glucose; 150 mg. per cent at the end of two hours and a value of 67 mg. per cent at the end of three hours. Gastric analysis showed no free acid after histamine. Five determinations of the amount of fat per dry weight of stool varied from 50 to 76.5 per cent. Serum calcium was 6.8 mg. per cent, serum phosphorus 2.9 mg. per cent and phosphatase 13 Bodansky units. Fluoroscopic studies of the gastrointestinal tract revealed no abnormality. Intestinal contents aspirated from the duodenum were normal for amylase and trypsin, but contained only small amounts of lipase activity.

Since Nov. 5, 1945, this woman has been given an average of 20 mg. of synthetic *L. casei* factor intravenously per day. Her reticulocytes began to increase on the third day and reached a peak value of slightly over 30.2 per cent. The red cell count has risen from approximately 2.5 to approximately 3.5 million cells, and the erythrocytes have now become hypochromic. The patient is much improved clinically, feels stronger, and has gained six pounds in weight.

M. W., white female, 32 years of age. This woman had always been in reasonably good health until the last trimester of her first pregnancy in 1935. At that time, and during the last two months of each of her four subsequent pregnancies, she became weak, pale and moderately short of breath. Each of her children was delivered in a different hospital and each obstetrician told her that she had a severe degree of anemia. Transfusions were given at the time her third and fourth children were born; liver extract was also administered but no careful hematologic study was made of her response to therapy. She was admitted to the St. Louis Maternity Hospital on Oct. 25, 1945, and shortly thereafter delivered her fifth child. It was found that she had a red cell count of slightly over 1 million cells and a diagnosis of

pernicious anemia of pregnancy was made. The patient and her husband, however, refused to let transfusions be given and the patient discharged herself from the hospital when an attempt was made to give her liver extract. On Nov. 14, 1945, she asked to be admitted to the St. Louis City Hospital because she was extremely weak and had by that time become convinced that further therapy was necessary. It is noteworthy that although this woman ate liver about twice a month, she ate only small amounts of any other form of meat. She regularly prepared a meat dish for her family each day, but usually chose to eat none herself. She did regularly drink three glasses of milk a day, eat one or two eggs a day, and usually some cheese. Her weight gain during this last pregnancy was normal.

Physical examination showed the patient to be listless, extremely weak, and very pale. There was a definite lemon yellow tint to her sclera and skin. No significant enlargement of lymph nodes was detected. The papillae of her tongue were not atrophic. Both lung fields were clear; the heart was not enlarged in size; rhythm was regular; a soft systolic murmur was audible over the whole precordium. Liver and spleen were not felt. Pelvic examination showed that the uterus was apparently involuting in a normal manner. Examination of the extremities revealed no abnormality. Reflexes were normally active and equal on the two sides; no sensory disturbances were detected.

Urinalysis was within normal limits. Hematologic study showed the red cell count to be 1,130,000, hemoglobin 5.5 grams, reticulocytes 7.4 per cent, white cells 10,000, platelets 89,300. Leucocytic differential was as follows: myelocytes 3, metamyelocytes 2, stabs 2, segmented neutrophils 53, lymphocytes 36, monocytes 4. There was marked anisocytosis and poikilocytosis of the red cells with definite macrocytosis. Three normoblasts were present for one hundred white blood cells. The hematocrit value was 16 per cent, the mean corpuscular volume 145 cubic microns, and the mean corpuscular hemoglobin concentration 36 per cent. Bone marrow removed by sternal aspiration was very cellular and was characterized both by an increase in the percentage of erythroid elements and a definite predominance of early erythroblasts and megaloblasts. The icterus index was 12 and the serum iron value 0.183 mg. per cent. Blood Kahn reaction was negative. A histamine refractory achlorhydria was present on Nov. 16, 1945, but 11 degrees of free acid were found thirty minutes after histamine on Nov. 27, 1945.

Daily administration of 20 mg. of *L. casei* factor intravenously was begun on Nov. 17, 1945. Within forty-eight hours, the patient experienced a sense of well-being which was quite remarkable. Her red cell count has risen rapidly so that a level of 3 million cells was attained on the fifteenth day after therapy was instituted. She has now been discharged from the hospital.

#### NOTES

\*Fermentation factor of Hutchings and associates.<sup>7b</sup>

\*\*Generously supplied by the Cerophyl Laboratories, through the courtesy of Dr. W. R. Graham, Jr.

\*\*\*Charcoal adsorption and elution, followed by superfiltrol adsorption and elution. This material had an activity of 1.6 mg. potency 40,000 units per gram; assayed with *L. casei*. As nearly as can be estimated at present, 1 mg. of potency 40,000 units is equivalent to from 0.25 to 0.5 mg. of the pure *L. casei* factor.

\*\*\*\*Two additional patients with Addisonian pernicious anemia are currently being given 2 mg. of synthetic *L. casei* factor intravenously per day. Reticulocytes began to rise in both patients on the fifth day of therapy; but the response is submaximal and the clinical improvement has not been dramatic.

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## LABORATORY METHODS

### A NOTE ON GASTRIC RETENTION IN ONE-HOUR, TWO-DOSE GLUCOSE TOLERANCE TESTS

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ONE of the most popular glucose tolerance tests at the present time is the one-hour, two-dose test devised by Exton and Rose in 1934.<sup>1</sup> The test as originally described is carried out by giving two 50 Gm. doses of glucose thirty minutes apart and determining the blood sugar at 0, 30, and 60 minutes. The criteria of a normal response include a fasting blood sugar within the normal limits of the blood sugar method employed, a rise in blood sugar which does not exceed 75 mg. in the 30-minute sample, a blood sugar level in the 60-minute sample which is less, the same, or does not exceed the 30-minute sample by more than 5 mg., and urine samples that are all negative to Benedict's test. The test is based on Allen's paradoxical law<sup>2</sup> which states that there is no limit of tolerance to glucose given by mouth to the normal animal but that diabetic animals exhibit definite limits of tolerance. The test also utilizes the phenomenon observed by Hamman and Hirschmann,<sup>3</sup> that after successive doses of dextrose given by mouth, the level of each successive rise in the blood sugar curve is lower than the preceding one. Since the original description of the one-hour, two-dose test, there have been several clinical studies made employing the test and criteria as originally described or using some modified criteria for the diagnosis of diabetes mellitus and other disorders of carbohydrate metabolism.<sup>4-9</sup> This report describes studies of the gastric retention of glucose following the one-hour, two-dose test.

Glucose tolerance tests were carried out in sixteen normal male medical students following the procedure of Exton and Rose.<sup>1</sup> After taking the 60-minute blood sample, the gastric contents were withdrawn by a stomach tube and analyzed for glucose. The time required for obtaining the gastric contents was generally about ten to twenty minutes. The venous blood sugar levels determined by the Myers-Bailey method<sup>7</sup> averaged 96, 143, and 139 mg. per 100 c.c. for the 0-, 30-, and 60-minute samples, respectively. However, the amount of glucose recovered from the stomach varied from 38 to 62 Gm. and averaged 50 Gm. There was no correlation between the blood sugar changes in the various subjects and the amount of glucose remaining in the stomach. It is apparent that although 100 Gm. of glucose were ingested in the two doses, less than 50 Gm. of sugar usually emptied from the stomach into the duodenum and correspondingly less than 50 Gm. were absorbed into the blood stream during the hour. This is consistent with our observations and those of others,<sup>4-6, 8, 9</sup> that the average blood sugar pattern is quite similar during the first hour, when either one dose or two doses of glucose are given. Many investigators have failed to give this fact proper attention and have been inclined to accept the two-dose test as a more accurate index of disturbed carbohydrate metabolism.

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The finding of more than 50 Gm. of sugar in the stomach at the end of one hour in the one-hour, two-dose test raises a serious question as to whether the administration of a second dose of sugar is of any advantage in glucose tolerance tests of the Exton-Rose type. Any superiority of the two-dose test over a one-dose test could be merely due to the more critical criteria that are applied to the two-dose test.

## SUMMARY

In sixteen normal adults subjected to a one-hour, two-dose glucose tolerance test (Exton-Rose type), over one-half of the administered sugar remained in the stomach at the end of one hour. A question of any advantage in administering two doses of sugar in such tests is raised.

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## PHOTOELECTRIC DETERMINATION OF BLOOD THIOCYANATES WITHOUT PRECIPITATION OF PROTEINS

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IN THE treatment of hypertension with potassium thiocyanate, frequent determinations of the amount of thiocyanate in the blood are important to maintain adequate therapeutic levels and to prevent possible toxic effects from overdosage. The colorimetric determination of thiocyanate has been based upon its reaction with a ferric salt to form a colored compound. The original method adapted by Schreiber<sup>1</sup> from the procedure of Leared<sup>2</sup> has been variously modified. One of the most widely used methods is that of Barker,<sup>3</sup> and modifications have been proposed by Crandall and Anderson,<sup>4</sup> Laviates, Bourdillon, and Klinghoffer,<sup>5</sup> Griffith and Lindauer,<sup>6</sup> Ravin,<sup>7</sup> and others.<sup>8, 9</sup> Molenaar and Roller<sup>10</sup> proposed a photometric method and Ginsburg and Benotti<sup>11</sup> have devised a method using the Evelyn photoelectric colorimeter which does not require the preliminary precipitation of the serum proteins. The method to be described was devised for use with the Klett-Summerson photoelectric colorimeter but

could easily be adapted for use with photoelectric instruments of other types. The advantages of this method and its accuracy as compared with previous methods will be discussed.

#### METHOD

##### Reagents.—

##### 1. Thiocyanate standard\*

(a) Stock—Dissolve 2.0 Gm. of C.P. KCNS in 1 liter of distilled water. Titrate this solution against 20 c.c. of a solution of C.P.  $\text{AgNO}_3$  (2.924 Gm./liter) plus 5.0 c.c. of concentrated  $\text{HNO}_3$ , using 1.0 c.c. of a saturated aqueous solution of ferric ammonium sulfate as an indicator. Calculate the dilution of the thiocyanate solution needed to make 20 c.c. of it equivalent to 20 c.c. of the silver nitrate solution. After making this dilution, check by another titration to be sure the potassium thiocyanate solution is exactly equivalent to the silver nitrate solution. This final stock solution contains 100 mg. of CNS ion per 100 c.c.

(b) Working standard—Dilute 10 c.c. of the stock standard to 100 c.c. with distilled water. This solution contains 10 mg. of CNS ion per 100 c.c.

##### 2. Ferric nitrate solution

Dissolve 50 Gm. of crystalline ferric nitrate in 500 c.c. of distilled water, add 25 c.c. of concentrated  $\text{HNO}_3$  and make to 1 liter with distilled water.

##### 3. Blank solution

Dilute 25 c.c. of concentrated  $\text{HNO}_3$  to 1 liter with distilled water.

**Calibration.**—In Klett-Summerson tubes mix 1.0 c.c. of working thiocyanate standard, 8.0 c.c. of distilled water, and 1.0 c.c. of ferric nitrate solution. Make this determination in triplicate, read after five minutes against a distilled water blank at zero, using a No. 54 filter (spectral range, 500-570 millimicrons), and record the average of the three readings as the *standard reading S*. Since the reagents are stable, a new standard reading need be determined only at intervals of several months or when a new set of reagents is made up. It will be shown in the subsequent discussion that there is direct proportionality between the readings with the Klett-Summerson instrument and varying concentrations of thiocyanate.

#### TEST

UNKNOWN	BLANK
4.0 c.c. distilled water	4.0 c.c. distilled water
0.5 c.c. serum	0.5 c.c. serum
Mix.	Mix.
0.5 c.c. ferric nitrate reagent, slowly with shaking	0.5 c.c. blank solution, slowly with shaking

Mix well, let stand five minutes and read in the instrument using the No. 54 filter.

**Calculations.**—Subtract the reading of the blank from the reading of the unknown to obtain the true reading of the unknown (*U*).

$$\frac{U}{S} \times 10 = \text{mg. of CNS ion per 100 c.c. of serum}$$

\*In the method described by Barker<sup>3</sup> there is an error which apparently has escaped detection by later writers.<sup>4</sup> According to Barker's directions, one dissolves about 1 Gm. of potassium thiocyanate in 800 c.c. of distilled water and then standardizes this against a solution of silver nitrate containing 2.9195 Gm. per liter, so that the final stock solution of thiocyanate contains 100 mg. of thiocyanate ion per 100 c.c. Obviously, a solution of potassium thiocyanate containing 1 Gm. per 800 c.c. is too weak to be made equivalent to the silver nitrate solution described and from such a solution it would not be possible to prepare a stock standard of the required strength.

## DISCUSSION

Since potassium thiocyanate is hygroscopic, an accurate standard cannot be prepared by weighing out the salt. Instead, it is necessary to make a solution stronger than the one required and to standardize this against a silver nitrate solution.

The calibration of the Klett-Summerson instrument having a special scale is simplified by the fact that the scale readings are directly proportional to the thiocyanate in varying strengths of standard. The calibration curve is a straight line except for concentrations approaching 20 mg. per cent as is shown in Fig. 1. Therefore, the use of a standard reading  $S$  obtained from readings on a standard solution containing 10 mg. of CNS ion per 100 c.c. is valid within the range of values usually encountered in blood thiocyanate determinations.

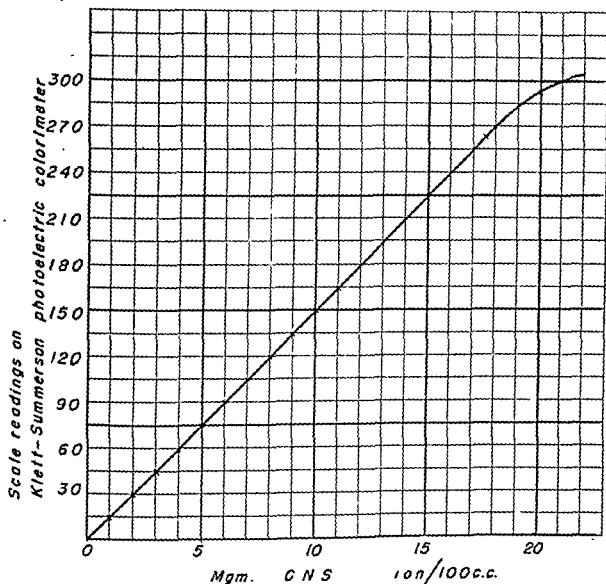


Fig. 1.—Calibration curve for Klett-Summerson photoelectric colorimeter with varying concentrations of thiocyanate standard.

Ginsburg and Benotti<sup>11</sup> employed as a ferric salt a solution of ferric chloride instead of ferric nitrate, since they found that in the proportions of one part of serum to two parts of reagent excessive turbidity was produced by the ferric nitrate reagent. With the method proposed in the present paper, this objection does not apply since the final dilution of serum is 1:10. Numerous observations have shown that any turbidity produced is minimal, is not progressive for periods up to one hour, and is, in addition, perfectly compensated for by the blank determination. The ferric chloride reagent has several disadvantages: First, The reagent itself has a distinct yellow color which precludes the use of a blank reading, except, as is proposed by Ginsburg and Benotti,<sup>11</sup> on serum

from each patient before any thiocyanate is administered. Not only would this procedure be time consuming and often not feasible in routine clinical laboratory practice, but, in addition, it disregards the thiocyanate normally present in human blood serum which is often not inconsiderable. A second disadvantage in the omission of a routine blank determination is that in jaundiced or hemolyzed blood a considerable error is introduced. In the proposed method neither jaundice nor hemolysis alters the accuracy of the final readings since the blank reading compensates for any altered serum color (see Table I).

TABLE I.—EXPERIMENTAL RECOVERY OF THIOCYANATE ADDED TO SERUM

SAMPLE	ANTICIPATED RECOVERY (MG.)	ACTUAL RECOVERY (MG.)	PERCENTAGE RECOVERY (PER CENT)	PERCENTAGE ERROR (PER CENT)
1	2.95	3.0	101.0	+1.0
2 (Red from hemolysis)	5.15	5.10	99.0	-1.0
3	5.3	5.3	100.0	0.0
4 (Red from hemolysis)	5.35	5.35	100.0	-0.0
5 (Red from hemolysis)	6.90	6.93	100.3	+0.3
6	10.23	10.33	100.9	+0.9
7	10.5	10.33	98.3	-1.7
8 (Red from hemolysis)	10.53	10.7	101.6	+1.6
9 (Bilirubin, 3.4 mg. %)	10.53	10.7	101.6	+1.6
10	13.6	13.6	100.0	0.0
11	15.2	15.0	98.6	-1.4
12	16.5	16.3	98.1	-1.9

Maximum per cent error, 1.9; Average per cent error,  $\pm 0.95$ .

A possible third disadvantage in the use of ferric chloride lies in the rapid fading of the color produced. According to Ginsburg and Benotti,<sup>11</sup> the color fades appreciably after five minutes and any delay in reading causes a considerable error. The ferric nitrate reagent, in contrast, produces a color which I have found to be constant for periods up to several hours. However, in my own experiments with the ferric chloride reagent I have had no difficulty with fading but have found the color produced to remain constant for at least one hour.

The accuracy of the method proposed is shown by the quantitative recovery of thiocyanate added to serum in varying amounts as is shown in Table I. Neither jaundice nor hemolysis interferes with accurate recovery.

Trichloroacetic acid has been employed as the precipitant in the majority of methods involving preliminary protein precipitation. Chesley<sup>12</sup> concluded, after a number of experiments, that either trichloroacetic or tungstic acid was satisfactory. Parallel determinations by the proposed method and a method

TABLE II. COMPARATIVE RECOVERIES OF THIOCYANATE WITH AND WITHOUT PRECIPITATION OF PROTEINS

SAMPLE	ANTICIPATED RECOVERY (MG.)	RECOVERY BY PROPOSED METHOD (MG.)	% RECOVERY BY PROPOSED METHOD (PER CENT)	RECOVERY ON TRICHLOR- ACETIC ACID FILTRATE (MG.)	% RECOVERY ON TRICHLOR- ACETIC ACID FILTRATE (PER CENT)
1	5.0	4.95	99.0	5.0	100.0
2	10.7	10.65	99.0	10.37	96.9
3	12.03	11.85	98.5	11.85	98.5
4	13.02	12.74	97.8	12.60	96.5
5	13.8	14.07	101.9	13.3	96.4
6	14.4	14.4	100.0	13.7	95.1

using a trichloroacetic acid filtrate were made on pooled serum containing known, measured amounts of potassium thiocyanate. The results are shown in Table II. The recoveries shown are representative of those recorded for a number of similar experiments and indicate for the proposed method an accuracy equal or even superior to that of methods employing protein-free filtrates.

## SUMMARY

1. A rapid, simple method for the photoelectric determination of thiocyanate in serum without precipitation of proteins has been described.

2. Thiocyanate added to serum in known amounts is recovered with an average error of approximately  $\pm 1.0$  per cent.

3. The advantages of the proposed method over previous methods have been discussed.

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## A COLORIMETRIC DETERMINATION OF PARALDEHYDE

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A METHOD for the determination of paraldehyde in biologic fluids, following its administration, is provided by the p-hydroxybiphenyl color reaction.<sup>1</sup> The recovery experiments herein summarized show that the convenient procedure described by Stotz<sup>2</sup> for the analysis of blood acetaldehyde can be applied with little modification to the determination of paraldehyde.

The color is developed by the following procedure (for additional details, see reference 2): To 1 c.c. of the solution containing between 0.2 and 2.5  $\mu$ g. paraldehyde, add 0.05 c.c. of 5 per cent copper sulfate; cool in an ice bath while 8 c.c. of concentrated  $\text{H}_2\text{SO}_4$  (specific gravity, 1.84) are added slowly with agitation. Deliver 0.2 c.c. of the p-hydroxybiphenyl reagent (1 per cent p-hydroxybiphenyl in 0.5 N NaOH) directly into the solution; disperse the precipitate by shaking, and allow the tube to stand for one hour at room temperature with occasional shaking. Then heat in a boiling water bath for one and one-half minutes, cool, and read the intensity of the color in a photoelectric colorimeter with a 565 filter. A reagent blank prepared simultaneously with 1 c.c. water is used to adjust the galvanometer to 100, and the concentration of paraldehyde in the unknown is obtained from a standard curve; the latter is prepared by developing and reading the colors, as previously described, with a series of solutions containing known amounts of paraldehyde.

Possible sources of error in the use of this reagent have been described by Barker and Summerson.<sup>3</sup> The development of a strong green color in the test upon the addition of the p-hydroxybiphenyl reagent results in erratic and inadequate color development and is usually due to the use of impure sulfuric acid. Inadequate cooling of the solution during the addition of the  $\text{H}_2\text{SO}_4$  may give rise to a lactic acid color reaction.

When the usual 1:10 tungstic acid filtrate of blood is further diluted ten times or more, the color can be developed directly with a 1 c.c. aliquot. The expected blood levels of from 2 to 25 mg. per cent paraldehyde<sup>4, 5</sup> can thus be determined by the analysis of 1 c.c. of a 1:100 blood filtrate, and larger amounts can be determined on more dilute filtrates. Complete recoveries were obtained by this procedure when from 2 to 100 mg. per cent paraldehyde were added to blood; without added paraldehyde, 1:100 blood filtrates gave no color in the test. A 0.1 c.c. sample of finger-tip blood deproteinized in 10 c.c. dilute tungstic acid (prepared from 0.2 c.c. of 10 per cent sodium tungstate and 0.2 c.c. of 2/3 N  $\text{H}_2\text{SO}_4$ ) provides a convenient filtrate for paraldehyde analysis.

Less than 2 mg. per cent paraldehyde cannot be determined satisfactorily by a similar analysis of an undiluted 1:10 blood filtrate, since this more concentrated filtrate gives some color in the absence of added paraldehyde. These smaller amounts, as well as higher concentrations, can be determined by the analysis of a 1 c.c. aliquot of a distillate prepared in the manner described by Stotz.<sup>2</sup> The distillation procedure eliminates the interference by other blood

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constituents, such as lactic acid, and is therefore more reliable than a direct color development on the diluted filtrate. Complete recoveries of from 0.2 to 3 mg. per cent added paraldehyde were obtained in 5 c.c. of distillate when an aliquot of a 1:10 filtrate of blood was diluted to 10 c.c. and distilled. The trace of acetaldehyde normally found in blood<sup>2</sup> is a limiting factor in the analysis of minimal quantities of paraldehyde, and this interference becomes detectable when the paraldehyde concentration is less than 0.5 mg. per cent. Complete recoveries of from 2 to 100 mg. per cent added paraldehyde were obtained in 10 c.c. of distillate when 1 c.c. of the 1:10 tungstic acid filtrate was diluted to 15 c.c. and distilled. Urinary paraldehyde can also be determined by the distillation method; a preliminary precipitation of an aliquot with phosphotungstic acid (25 c.c. of 25 per cent phosphotungstic acid per 100 c.c. urine) and removal of the precipitate avoid excessive foaming during the distillation.

TABLE I. RECOVERY OF PARALDEHYDE ADDED TO BLOOD

PARALDEHYDE ADDED TO 1 C.C. BLOOD ( $\mu$ G)	PER CENT RECOVERY	
	DISTILLATION METHOD	DIRECT METHOD
2	123	--
3.5	110	--
5.5	101	--
7	98	--
15	101	--
20	99	--
25	95	--
30	87	110
70	100	100
150	100	104
200	94	102
300	99	100
400	99	95
500	102	102
750	97	97
1,000	95	98

The paraldehyde was added to the water with which the blood was laked during the preparation of a protein-free filtrate, and the latter was analyzed for paraldehyde by both the distillation and direct methods.

Paraldehyde was found to be quite stable in whole blood or in a tungstic acid filtrate during storage in a stoppered tube at 4° C.; less than 10 per cent loss occurred in three weeks. This method will not distinguish between acetaldehyde and paraldehyde, but the blood level of acetaldehyde is normally from 0.02 to 0.05 mg. per cent and would not be expected to exceed 2 or 3 mg. per cent even in acute alcoholism.

#### SUMMARY

A procedure is described for the determination of paraldehyde in biologic fluids by means of the p-hydroxybiphenyl color reaction.

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## THE J. S. B. STAIN FOR BLOOD PARASITES

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**A**LTHOUGH stained blood films are almost universally used for the demonstration of such parasites as the malaria parasites and Trypanosomes, no one method has received general acceptance. That this is true is evidenced by the new techniques, or modifications of old ones, which are continually appearing in print. The chemical reactions involved in the staining of cells are still largely unknown, and staining methods are still largely empirical.

Most of the commonly employed blood stains belong to the Romanowski group, so called because the Russian, Romanowski, devised the parent of them all in 1891. He used a combination of methylene blue and eosin which has been modified in different ways by numerous workers since but fundamentally improved by only a few. Of such modifications, Giemsa's is generally considered the best. Nevertheless, even this method has certain disadvantages, for it is rather slow and the stain is costly and difficult to prepare.

It is probable that in the United States, Wright's is more widely used than Giemsa's method. It gives excellent differentiation, is faster, and is less expensive. However, it is less uniform in the quality of results and does not keep well in hot climates.

In the United Kingdom, Leishman's modification is very popular. It is similar to Wright's in the manner of use and probably is more consistent in the quality of staining. It is, however, very difficult to obtain in the United States, and films stained with Leishman's are said to fade more readily than those stained by Wright's or Giemsa's technique.

Recently a new method has been introduced by Field (1940) which has been widely adopted because of its speed. By its use a thick smear can be stained in as little as ten seconds, and, provided drying can be hastened by placing in an incubator or current of hot air, it is ready for examination very soon afterward. Thus this method has a great advantage for a busy physician or technician who must make his diagnosis as soon as possible. But Field's stain is of little value for thin smears and does not give preparations which in any way equal those obtained by the older processes just mentioned.

This paper concerns a staining method which was devised and very recently reported by Singh and Bhattacharji (1944) and which seems superior to all other Romanowski staining processes wherever results of high quality, quickly obtained, are desired. We have used the method quite extensively and have found it to possess these advantages:

1. It is easily prepared from ordinary medicinal methylene blue and eosin.
2. It is relatively inexpensive.
3. It keeps well under different climatic conditions.
4. It is extremely fast (for thin smears, eighty seconds; thick films, thirty seconds).
5. It is equally good for both thick and thin smears, which may be stained together on the same slide.

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6. Solutions used for staining do not have to be made up each time, since they deteriorate little on standing.

7. Blood cells and parasites are clearly and brilliantly differentiated.

8. Results depend less on the pH of the diluting agent than with other Romanowski stains (especially Giemsa's).

Since the original article was published in India and may not be available to many workers in this country, directions for the preparation and use of the stain are given here. Certain changes which the originators of the stain have recommended since publication of their first paper have been included.

Two solutions are used. Solution I is made up from the following ingredients:

Medicinal methylene blue	0.5 Gm.
Potassium dichromate	0.5 Gm.
Sulfuric acid (1 per cent)	3.0 c.c.
Water	500.0 c.c.

"Dissolve the methylene blue thoroughly in 500 c.c. of water. Add the 1 per cent sulfuric acid, mix thoroughly, and then add the chrome salt. A heavy amorphous purple colored precipitate of methylene blue chromate forms. Heat in an autoclave at a temperature of 100° to 109° C. and a pressure of 0 to 5 pounds for three hours. At the end of this period, the solution turns blue which indicates almost complete polychroming. If the color remains greenish, further heating for another hour or so is required. If the temperature is allowed to rise above 110° C., the oxidation of methylene blue may be carried too far and the solution will turn a violet purple.

"When the solution has turned deep blue after three hours' boiling, allow it to cool at room temperature. Then add 10 c.c. of 1 per cent potassium or sodium hydroxide solution, drop by drop, very gradually while constantly shaking the flask. After the total amount of alkali has been added, transfer half of the contents of the flask into another of the same capacity and continue shaking for fifteen minutes more. Transfer the contents of the flasks into each other. In this way the precipitate will gradually get dissolved and the solution will turn deep blue with a violet iridescence. Leave it at room temperature for forty-eight hours for the solution to mature; afterward filter through a soft filter paper. . . . The solution will improve in staining qualities with age.

"Solution II. This is readily prepared by dissolving 1 Gm. of water-soluble eosin in 500 c.c. of tap water. A freshly prepared eosin solution may not yield as satisfactory a stain as one which has turned deep red after some use.

"Solutions I and II should be kept in wide-mouth stoppered jars, 1½ inches in diameter by 3½ inches in height, and set aside for forty-eight hours to mature. These keep well for several months, but in Solution I a thin golden yellow scum is likely to form on its surface due to a slight precipitation of the dye. This does not, however, interfere with staining, and the staining power of the solutions does not deteriorate with age."

Directions for using the stain are given as follows:

"Thick and thin smears taken on the same slide can be easily stained.

1. Fix the thin smear by dipping that part of the slide in a jar containing methyl alcohol for a minute or two.

2. Dry thoroughly, preferably by waving the slide in the air.

3. Immerse the whole slide in Solution I for thirty seconds.

4. Wash in a jar containing acidulated tap water (pH 6.2 to 6.6). With tap water in Delhi (pH 7.6, indicator, bromo thymol-blue) approximately 50 mg. of sodium dihydrogen phosphate or 5 drops of 5 per cent acetic or citric acid solutions for each 100 c.c. of water are necessary.

5. Stain with Solution II for one second.

6. Wash in the same jar (4) for four seconds.

7. Immerse in Solution I again for thirty seconds (3).

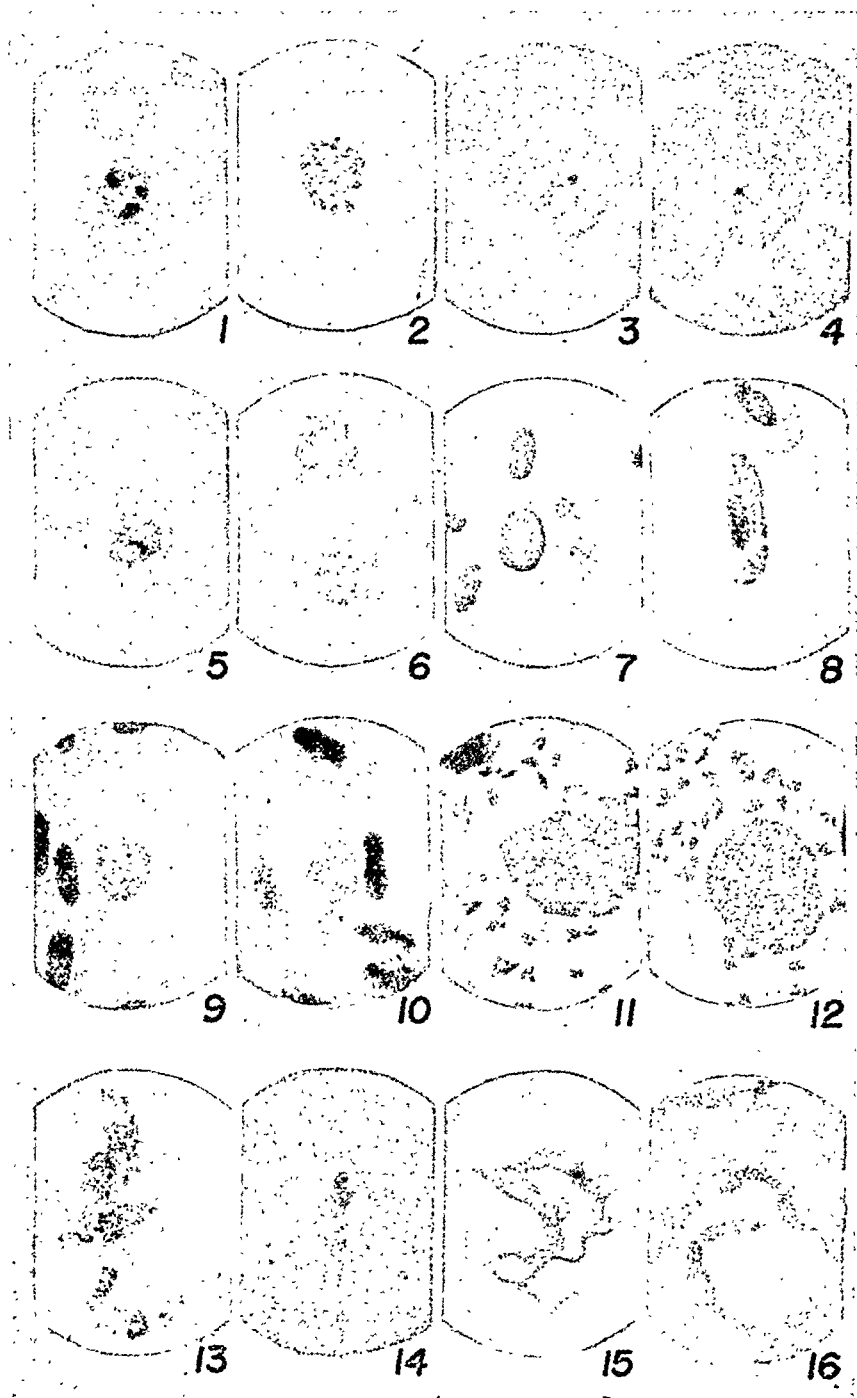


Fig. 1.—(See opposite page for legend.)

8. Wash as above for ten seconds or till the smear gives a pink background (4).

9. Dry and examine.

#### Thick Smear Alone

1. Immerse the slide in Solution I for ten seconds.

2. Wash in a jar containing water acidulated (pH 6.2 to 6.6) for two seconds.

3. Stain with Solution II for one second.

4. Wash in the same jar (2) for five seconds.

5. Immerse in Solution I again for ten seconds.

6. Wash as above for two seconds or till the smear gives a pink background (2).

7. Dry and examine.

The total time taken for staining a combined thick and thin smear is one minute and twenty seconds, and for a thick smear, only thirty seconds."

We have found that all the common blood parasites for which Giemsa's or Wright's stains are usually used can be demonstrated equally well by the method just outlined. Altering the pH of the medium, and particularly of the water used for washing the slide, appears to make very little difference in the results obtained. However, an acid pH does tend to give the erythrocytes a rather reddish or orange appearance, while a more alkaline reaction results in giving them a gray or more bluish cast.

Since Giemsa's method seems to give the best results for most of the blood protozoa, it seemed worth while to compare the two methods as exactly as possible. Accordingly, smears were prepared of a number of species of Plasmodia, several species of Trypanosomes, and of *Leishmania donovani*. Some of each parasite were then stained by each method. These were critically compared under the microscope, and a series of photographs was made as shown in Fig. 1. The photographs in the first and third columns were made from slides stained by the Giemsa technique and those in the other two columns show the same species of parasite stained by the J. S. B. process. It will be noted that the two techniques give equal differentiation. Actually the color characteristics of both parasites and blood cells are similar, whichever the process used, but black and white photographs cannot give clear evidence of this fact.

To determine how the two stains compared with respect to fading, slides of *Plasmodium malariae* prepared by each process were divided into four groups. These groups were similar in number, and the slides in each were comparable in every respect. One of the groups served as a control, and the other three were subjected to the radiation from a General Electric sunlamp of 450 watts. The ultraviolet light from this lamp is said to equal five times the ultraviolet

Fig. 1.—The microphotographs in the first and third columns were stained by the Giemsa technique; the others were stained by the J. S. B. method. In each case comparison is easy, since the same species of parasite is shown stained by each of the two methods. Magnification is X1350.

1, A schizont of *Plasmodium knowlesi*. Giemsa, at pH 7.4. (AMM negative 90231.) 2, A segmenter of the same species. J. S. B. at pH 6.5. (AMM negative 90231.) 3, A ring form of *Plasmodium vivax*. Giemsa at pH 7.4. (AMM negative 90227.) 4, A slightly larger stage, also vivax. J. S. B. at pH 6.5. (AMM negative 90228.) 5, A large trophozoite of *Plasmodium malariae*. Giemsa at pH 7.4. (AMM negative 90262.) 6, Two parasites (trophozoites) of *Plasmodium malariae*. J. S. B. at pH 6.5. (AMM negative 90266.) 7, *Plasmodium elongatum*. Giemsa at pH 7.4. (AMM negative 90240.) 8, same species. J. S. B. at pH 6.5. (AMM negative 90247.) 9, *Plasmodium relictum*. Giemsa at pH 7.4. (AMM negative 90277.) 10, o a female gametocyte. J. S. B. at pH 6.5. (AMM negative 90255.) 11, spleen smear (hamster). Giemsa at pH 7.4. (AMM negative 90215.) 12, *Trypanosoma equiperdum*. J. S. B. at pH 7.4. (AMM negative 90210.) 13, *Trypanosoma equiperdum*. Giemsa at pH 7.4. (AMM negative 90226.) 14, *Trypanosoma equiperdum*. J. S. B. at pH 6.5. (AMM negative 90224.)

radiation of full sunlight at a distance of thirty inches, so that the slides, which were placed only fifteen inches away, received twenty times as much ultra-violet radiation as from the sun in an equivalent time. Group I was exposed 9.3 hours; Group II, 35.4 hours; and Group III, 53 hours. Sunlight equivalents would be 186, 708, and 1,060 hours, respectively.

It was found that some fading occurred in all the radiated groups, and that slides stained with the J. S. B. stain suffered somewhat more than those prepared by Giemsa. Furthermore, slides on which stains buffered with the lower pHs were used faded more than those to which stains of more alkaline pH were applied. It is therefore clear that staining in an alkaline buffer gives more lasting preparations; yet even on those slides which faded the most, parasites and leucocytes were still easily visible, although the reddish components of the dyes had been largely bleached out.

#### CONCLUSIONS

The J. S. B. stain, recently introduced by Singh and Bhattacharji, has been tested and been found superior in most respects to any of the other commonly used processes for the staining of blood and blood protozoa. The technique is simple and the staining process can be completed in less than two minutes for thin smears and in less than one minute for thick films. The staining solutions are not difficult to make up, are relatively inexpensive, and keep well for weeks or months, even in hot weather. Preparations stained by this process appear very much like those made by Giemsa's method, cytoplasm and chromatin of blood cells and parasites being differentiated with equal clearness and having similar color values. J. S. B. preparations are somewhat less resistant to fading but will stand much more exposure to light than they would ordinarily receive.

Thanks are due T/4 Ruth Cutter Burkenbilt for assistance in this study.

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#### Erratum

In the article by Andres Goth, entitled "The Antitubercular Activity of Aspergillie Acid and Its Probable Mode of Action," which appeared in the November issue of the JOURNAL, the sentence beginning in the sixth line from the bottom on page 900 should read: "However, these red colonies failed to grow as well as the controls to which no aspergillie acid had been added."

# INDEX TO VOLUME 30

## AUTHORS INDEX

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